ON THE EFFECT OF EMBRYONIC AND VARIOUS GLANDULAR EXTRACTS UPON THE GROWTH OF A MOUSE SARCOMA CULTIVATED *IN VITRO*: A CYTOLOGICAL STUDY

by

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The purpose of this investigation was to observe the general appearance, behaviour, and rate of growth of a mouse sarcoma when cultivated *in vitro* in embryonic and various glandular extracts, and also the cytological changes which occurred during growth and differentiation. The authors wish to acknowledge their indebtedness to Dr. Kellaway, Director of the Walter and Eliza Hall Institute, Melbourne, for providing the original strain of mouse sarcoma.

MATERIAL AND TECHNIQUE FOR TISSUE CULTIVATION.

A round-celled sarcoma was employed; all cultures were made by the usual hanging-drop method, and were grown *in vitro* in the following media:

(a) Testicular extract from a tumour-bearing mouse.
(b) Testicular extract from a normal healthy mouse.
(c) Tumour extract.
(d) Chicken embryonic extract.

Plasma obtained from adolescent chicks was used throughout as a supporting medium.

Preparation of Extracts. All extracts were prepared by a mincing of the given tissues in sterile Ringer's solution with the aid of a pair of de Wecker's iris scissors. The mixture, after standing a short while, was centrifuged for several minutes; the supernatant fluid was pipetted off and then used as the extract. When embryonic tissue juices were employed, the embryos used for making the extract were usually from 7–10 days old.

In the case of the chick plasma, the blood was collected into paraffin-coated Wassermann tubes, previously cooled at 0°C. The supernatant plasma obtained

after centrifuging was kept in paraffined tubes in a refrigerator, since by this means the plasma may be kept fluid for several successive weeks.

When selecting the pieces of sarcoma to be cultivated we attempted to obtain tumour fragments that were comparatively free from the tissues of the host. These fragments were then placed upon a sterile cover-glass, equal parts of plasma and extract were added with a sterile pipette, and the fluids were then mixed together with the aid of a cataract knife. The preparation about to be cultured was then inverted over the hollow ground slide, sealed in the usual manner, and incubated at 38°C.

For a study of the cytoplasmic inclusions, cultures were stained *intra vitam* with James Green B and neutral red. In order to provide fixed preparations, cultures were first immersed for several minutes in a warm Ringer's solution, so as to prevent coagulation of the plasma. The explants were either treated with osmic vapour or fixed in a 2% formalin solution, and stained by the Haidenhain iron-haematoxylin method.

To carry out investigations *in vivo*, the explants were placed upon a warm stage kept at incubation temperature.

During this investigation 28 cultures were made, employing testicular extract from sarcomatous mice, and the same number were prepared in which testicular extract from healthy normal mice were used.

Twenty-four cultures were made employing the embryonic medium, while 21 were prepared of tumour extract.

GENERAL APPEARANCE OF GROWTH *IN VITRO*.

Usually many cells may be detected migrating from the tumour fragment along the cover-slips or upon the surface membrane of the hanging drop after 14–15 hours' incubation *in vitro*. A more detailed examination of these cellular elements under the powers of the microscope reveals that the zone of growth is composed of three cell-types. These are so strikingly different from each other in both their morphology and behaviour as to attract immediate attention (see figures 5–8). The first type, which greatly predominates in numbers in actively

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EXPLANATION OF PLATE 1.

All explants were fixed after three days' cultivation *in vitro* in a 2% solution of formalin or by osmic vapour, and stained with Heidenhain's iron haematoxylin. Microphotographs 1-4 were taken with the same magnifications.

Fig. 1. Culture *in vitro* of a round-celled mouse sarcoma, fixed after three days' growth in a medium of embryonic extract.

Fig. 2. The same, fixed after three days' growth *in vitro* in a medium of tumour extract.

Fig. 3. Explant *in vitro* fixed after three days' growth in a medium of testicular extract from a tumour bearing mouse. Note the extensive cellular proliferation.

Fig. 4. The same, fixed after three days' growth *in vitro* in a medium of testicular extract from a normal healthy mouse.

Fig. 1.

Fig. 2.

Fig. 3.

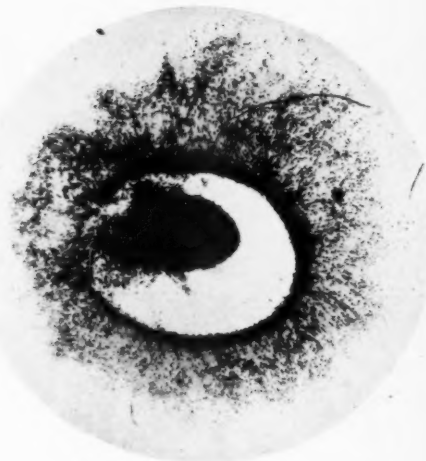

Fig. 4.

PLATE 1.

growing cultures, corresponds to the pseudo-fibroblasts already described by several authors (4, 6, 7, 8, 15). The second type represents the sarcoma cells proper, and the third type is similar to the elasmatocytes or amoeboid cells, first described by Lewis and Gey (14).

These three cell-types were observed in all cultures of sarcomata, and their cytological structure and behaviour will be the subject of discussion in a later chapter.

That the appearance of the zone of growth of explants depends upon the culture medium employed is agreed upon by several authors, among whom is Walton (19). The latter recently investigated the rate of growth of mammalian tissues *in vitro* in various extracts, which he showed are specific in their action upon the growth of cells, some cells being inhibited by one extract and stimulated by another. A comparative examination of the effects of the various culture media employed during this investigation upon the growth of the explants confirms the recent conclusions of these authors. If a culture *in vitro* of a round-celled sarcoma, fixed after a 3 days' growth in a medium of testicular extract from a sarcomatous mouse, be examined and compared under the powers of the microscope with a tumour fragment cultivated for the same period and at the same temperature in a medium of testicular extract from a normal, healthy rodent, a vast difference in the rate of growth of the two cultures will immediately be detected (see figures 3 and 4 for comparison); more detailed observations will reveal that the zone of growth, total emigration of cells, in the former culture is much greater than in the latter; in fact, the whole explant exhibits a much more compact appearance and more mitotic phases, all of which is suggestive of a much more rapid growth. Bearing in mind the recent work of these investigators, showing that different culture media vary in their action upon the growth of cells cultivated *in vitro*, these results strongly suggest that the testicular extract from the sarcomatous mice has a more stimulating effect upon the tumour cells of the culture than that of normal mice. Similarly, when sarcoma fragments which have been cultivated separately in embryonic and tumour extracts for the same period are studied, observations again show a vast difference in the rate of growth between the two cultures (see figures 1 and 2). The former explant shows a greater radial outgrowth, more cellular outwandering, and more mitosis than cultures grown in embryonic media. Again it is suggested that the tumour extract has a more stimulating effect.

GENERAL CYTOLOGY OF CULTURES.

Although the general appearance and behaviour of various sarcomata when cultivated *in vitro* has recently been the subject of much study by several investigators, a great deal of controversy still exists among these workers concerning the cytological structure and character of the cells composing the general

growth of the cultures, and as their conclusions are not altogether in accord with our own observations, we have considered the subjects deserving of a further investigation. A morphological survey of the various cellular components as seen in preparation fixed after a three days' growth *in vitro* will now be made.

MORPHOLOGICAL STRUCTURE OF CELLS.

Pseudo-fibroblast cells conforming to this type vary greatly in form, but the majority may be said to be spindle-shaped; some, however, are oval or fan-shaped, while others assume a triangular outline. Others may become very elongated, and send out long protoplasmic strands, which intermingle with one another, often giving the appearance of a cytoplasmic network. This phenomenon is best seen among the cells composing the periphery of the explants. A glance at figures 5–8 will show that these cells are very variable in size. The nuclei of the elongated pseudo-fibroblasts are oval, while those in the more fusiform types are rounded (see figure 7), and are densely packed with chromatin. Multinucleated cells are occasionally detected, which are generally triangular in structure, and appear to be exclusively of the fibroblast-like type (see figure 6). The cytoplasm, as seen in these fixed preparations, has a vacuolated appearance, which may be due to fixation (see figures 5–8).

Malignant Cells. The tumour cells within the explant appear to be spherical and oval in shape. Around the periphery of the cultures these cells, which have migrated, have become slightly more elongated, and thus resemble the fibroblast-like elements in shape (see figure 7). The possibility that the pseudo-fibroblasts are mere morphological variations of the malignant cell, and not generically separate types, immediately presents itself, and will be discussed in another section of this paper. The majority of these round tumour cells possess a large spherical nucleus, densely packed with chromatin; their protoplasm presents a slightly vacuolated appearance (see figure 7).

Clasmatocytes, or amoeboid cells, which appear in numbers in all cultures, present a striking contrast to the tumour cells and fibroblast-like elements; many pseudopodia appear to be thrown out simultaneously, making them appear very irregular in outline. The nuclei, which contain numerous chromatin granules, are generally distorted, and very often project into the pseudopodia (see figure 8). These amoeboid cells vary considerably in size, and their cytoplasm contains numerous small vacuoles.

By *direct illumination.* In order to observe the behaviour of the explants *in vitro*, 3 days' old cultures were placed upon a warm stage kept at incubation temperature, thus permitting them to be studied at leisure. By employing this method the outline and internal structures of the living cells are shown quite clearly. The protoplasm appears clear, transparent, and homogeneous. The

Fig. 5.

Fig. 6.

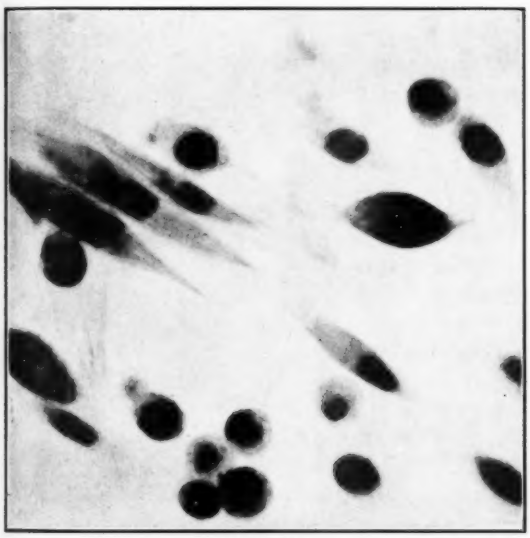

Fig. 7.

Fig. 8.

EXPLANATION OF PLATE 2.

Figs. 5 and 7. Microphotographs taken round the periphery of an explant, showing the transitional stages of the malignant cell into the fibroblast-like element. Several intermediate forms may be seen.

Note the darkly staining nuclei of the cells; also observe the reticulate structure of the cytoplasm.

Fig. 6. Showing large polynucleated cell; seven or more darkly staining nuclei may be seen.

Fig. 8. Microphotograph showing an amoeboid cell or clasmatocyte. Note the distorted appearance of the nucleus, which projects into the pseudopodia, also observe the vacuolated cytoplasm as well as the oval nuclei of the pseudo-fibroblasts.

nuclei of the cells are fairly distinct, and are generally observed to contain several dull greyish granules, the chromatin masses. A variable number of highly refractive cytomicrosomes (the mitochondrial-lipoidal droplets) and neutral red granules may also be detected undergoing active movements within the protoplasm of the living cells. In order to observe closely the appearance and behaviour of the cellular elements *in vitro*, the cells composing the periphery of the growth were chosen; in this region they appear more isolated, and their cytological characters may be studied to advantage. Observation of a fully-developed pseudo-fibroblast under an oil immersion lens shows the cell to be slightly more elongated in the direction in which it happens to be migrating; generally at each end of the cell delicate finger-like processes may be seen adhering to the cover-slip.

The tumour cells within the explant appear to be oval or round in shape, but those present in the peripheral region tend to become more elongated. A closer examination of these cultures showed many intermediate forms between the oval malignant cell and the pseudo-fibroblasts, such as were observed in the fixed preparations; and as this investigation *in vivo* affords an excellent opportunity to decide upon the nature and origin of these cells in question, more prolonged observations were made, in which it was actually possible to detect the oval tumour cells gradually transforming into the fibroblast-like elements. The malignant cells, as they tend to become more elongated in form, send out thin, needle-like processes from one or both ends, and thus come to assume the shape and appearance of the pseudo-fibroblasts so characteristic of the sarcomata when cultivated *in vitro*.

The behaviour of the nucleus during the actual transitional stages of the malignant cell was of interest, as the round monocyte, together with its large spherical nucleus, slowly lengthened out, its nucleus gradually became fusiform, and by the time complete differentiation had occurred the nucleus of the newly-formed pseudo-fibroblast had become elongated in shape.

When studied *in vivo* the amoeboid cells or clasmatocytes present a remarkable contrast when compared with the fibroblast-like elements and tumour cells. They are more active in their movements; pseudopodia are regularly thrown out and quickly withdrawn, making their movements remarkably motile. Observations upon fixed preparations showed their nuclei to be very constricted, giving the appearance of an artefact (see figure 8), but *in vivo* the nucleus was often seen extending in a distorted manner into the pseudopodial processes.

The most conspicuous of the cytoplasmic inclusions, when studied *in vivo*, are the mitochondria, and these highly refractive granules appear in varying quantities in all cells composing the cultures. They may often be seen scattered somewhat irregularly through the cell protoplasm, and they generally occur in larger numbers around the nucleus and periphery of the cell.

In a previous paper one of us pointed out that this effect, such as is seen in many cells, is a surface tension phenomenon dependent upon the phosphatidal nature of these bodies (9). The mitochondria in the living cell may be seen in the form of rod-like and spherical bodies undergoing marked Brownian movements within the transparent protoplasm. Occasionally a rod-shaped mitochondrium may come to rest and constrict in the middle, until finally two small, rounded bodies are formed, which later elongate, and finally give rise to the original rod-like structure. Strangeways and Canti (18) recently carried out a striking investigation upon the behaviour of mitochondria in the living cell *in vitro*. They describe these bodies as undergoing "a slow, snake-like movement" within the cell-protoplasm.

Mitochondria are more numerous in the pseudo-fibroblasts than in the clasmatocytes, although in the latter type of cell they appear to be undergoing more violent movements, owing to the continuous turmoil of the cell. It is very fascinating to observe the mitochondria and other refractive cell-inclusions being jostled to and *fro* in the streaming transparent protoplasm of the pseudopodia. The mobility of these cell-granules is dependent largely upon the temperature at which the explants are growing; when the temperature is lowered they become less active, and as the temperature rises the rapidity of their movements increases. When stained *intra vitam* with a 1 : 10,000 solution of Janus green B, the mitochondria coloured a brilliant green. The role these granules play in cell metabolism is at the present time a matter of controversy, but the work of recent investigators (1, 10, 11, 12, 16, 17) indicates that they are of an enzymatic nature.

The lipoidal globules, which occur in larger numbers in the clasmatocytes than in any of the other cellular elements, present a brilliant picture when observed *in vivo*. Like the mitochondria, they are highly refractive, but appear less active in their movements. These droplets, which vary greatly in size, move about in little groups within the protoplasm of cells, and generally accumulate at one side of the nucleus. Their movements depend upon the activity of the cell, as occasionally little groups of these refractive globules get caught up by a protoplasmic current, separate out, and become distributed throughout the cell-cytoplasm. They appear more abundant in cultures which have been grown *in vitro* in a medium of either embryonic or testicular extracts. The manner in which these bodies arise is at present a matter of debate. Cowdry (5) holds that the cell-mitochondria may under certain conditions transform into droplets with pronounced fatty and lipoidal properties. When exposed to osmic vapour they turn black in colour.

The third type of granules are those which colour when stained *intra vitam* with neutral red. A great variation in staining reaction is shown. Some of these protoplasmic inclusions absorb the stain rapidly, and become a bright red, while

others hardly colour at all, and give a faint yellowish tinge. They are all spherical in form, and are seen scattered irregularly throughout the cytoplasm; sometimes they accumulate in little groups around the periphery of the cells, and appear less active than the mitochondria. The rôle these bodies play in cell metabolism is not yet determined.

Cells undergoing mitosis are well shown by means of direct illumination. In order to observe this phenomenon 3 days' old cultures were studied upon the warm stage. The cells composing the periphery of the explants were selected, owing to their isolated condition. A cell which is about to undergo division withdraws its pseudopodia and assumes a more or less spherical shape. The nucleus is fairly clear; during the prophase it appears as a dull greyish oval body. As mitosis proceeds the chromosomes may be faintly distinguished. The cell-granules appear to be undergoing more rapid movements than they do during the resting stage. During the anaphase their refractive bodies aggregate at the opposite poles of the cell, and later appear to distribute themselves more or less evenly among the newly-formed daughter cells.

A comparative examination of the cells grown in the testicular extracts shows proportionally more mitoses than those cultivated in the embryonic and tumour juices.

Multinucleated cells are occasionally detected when examining preparations fixed after several days' growth *in vitro*. They contain generally from 7–20 nuclei (see figure 6), which vary in size. We are unable to indicate how the polynucleated cells arise, as they have not been observed *in vivo*. They are polyhedral in shape, and their appearance seems to suggest that they are derived from the fibroblast-like elements.

DISCUSSION.

The results of this investigation have shown that the cultivation of tissues *in vitro* affords a valuable means of estimating the effect of various media upon the growth of the cells. Until recently the mechanism which governs the activity of the tissues within the organism have remained unknown, and it was uncertain whether the cells possessed in themselves some spontaneous activity, or whether they were entirely dependent upon the physico-chemical conditions of the medium.

The work of recent authors (19, 2), together with our present investigation, have conclusively shown that the rate of growth and activity of tissue cells is dependent largely upon the composition of the medium.

Carrel (2), who has studied this phenomenon in great detail, has successfully shown that the growth of cells when cultivated *in vitro* remains constant in an unchanging nutrient medium, and varies with modification in the composition of the medium, and he further infers that cells grown *in vitro* in different con-

ditions of activity will soon multiply at the same rate when placed in similar media. As we have already shown, cell proliferation is more active when the neoplastic tissue is cultivated in a medium of testicular extract from sarcomatous bearing mice than in a medium composed of testicular extract from healthy normal mice; similarly, it has been shown that embryonic juice does not so readily induce cell multiplication as the tumour extract.

Although it has been quite definitely established that the composition of the medium affects the rate of growth of tissue cells, little is at present known concerning the actual nature of the substances in the media which are directly responsible for active cellular proliferation. The advantages of tissue culture as a cytological technique are also admirable, as tissues grown in this manner do not lose their original characteristics. The individual cells are isolated, thus permitting their morphological and cytological characters, as well as their intracellular movements to be studied at leisure within the living cell.

A review of the recent literature dealing with tissue explantation will show that the cultivation *in vitro* of various sarcomata has been the subject of much study by several authors (6, 13), but nevertheless a great deal of controversy still exists among these workers concerning the behaviour and cytological structure of the cells composing these cultures. Few of these authors, however, have availed themselves of the opportunity of observing the behaviour of these cells *in vivo*, most of their conclusions being based upon preparations fixed after various periods of growth *in vitro*. Lambert and Hanes, for example, recognized in cultures of mouse and rat sarcoma two types of cells: a long spindle-shaped cell (which corresponds to the pseudo-fibroblasts described in our own cultures), and a second type, which in actively growing cultures is characterized by its extreme irregularity in shape, due to numerous pseudopods, which project from the cell in a most bizarre manner. These authors state further that these two cell-types are merely morphological variations, and not generically types.

Later, Lewis and Gey (14), when observing the appearance of a mouse sarcoma cultivated *in vitro*, describe two distinct types of cells migrating from the explants. The first type corresponds to the amoeboid cells first detected by Lambert and Hanes, and which they claim to be true clasmatocytes. The second oval type represents the sarcoma cell proper. Fell and Andrews (6), who recently carried out a cytological study *in vitro* of Jensen's rat sarcoma, describe two types of cells present in their cultures, which occurred in about equal numbers, cells resembling fibroblasts and smaller wandering cells, similar to the clasmatocyte described by Lewis and Gey. In concluding, these authors suggest that the wandering cells, or so-called clasmatocytes, may be the malignant component of the sarcoma, while the fibroblasts represent the stromal elements. These wandering cells, or clasmatocytes, figured by these several authors in active-growing cultures correspond in appearance and behaviour to

the amoeboid cells detected in our own cultures. The question naturally arises as to the origin of these bodies. When examining paraffin sections of sarcamatous tissue it is very difficult to detect these highly amoeboid cells, as they are so closely packed together as to entirely obliterate their cytoplasmic characters.

That the pseudo-fibroblasts, as is suggested by Lambert and Hanes, should transform into the so-called clasmatocytes seems highly improbable. These amoeboid cells present such a striking contrast when compared with the fibroblast-like elements, and, moreover, a study of preparations, fixed after various periods of growth *in vitro* or actual observations *in vivo*, give no indication of any such transitional forms. Moreover, in cultures of young rat and mouse mesenchyme cells, similar [redacted] in general appearance and behaviour, may be detected migrating from the explants after several hours' cultivation; the evidence in hand at the present moment strongly suggests that these elements are stromal in origin.

Bearing in mind our conclusions that the pseudo-fibroblasts are merely morphological variations of the tumour cell proper, still more striking are the recent observations of Carrel and Ebeling (3). These authors succeeded in isolating a pure strain of fibroblasts from a Jensen sarcoma, and they found that these cells give rise to tumours on transplantation into animals. Their experiment demonstrates the malignant character of the pseudo-fibroblasts, which are in reality transformed tumour cells. These investigators have also detected several differences between the fibroblasts of a Jensen sarcoma and normal fibroblasts. They find that the true fibroblasts are unable to multiply when in blood serum, while the Jensen fibroblasts are enabled to proliferate in such a medium. They further observe that the sarcoma fibroblasts differ from the true rat-fibroblasts observed in pure cultures by the refringement aspect of the general cytoplasm and its coarseness.

SUMMARY.

Cultures *in vitro* of a round-celled sarcoma were made in the following media: Testicular extract from tumour-bearing mice; testicular extract from normal, healthy mice; tumour extract; embryonic extract. Plasma from adolescent chicks was employed throughout as a supporting medium.

1. Cultures grown in testicular extract from tumour-bearing mice showed more extensive outwandering, more mitotic phases, and much more rapid growth than cultures grown in testicular extract from normal healthy mice.

2. Similarly, cultures grown in a medium of embryonic extract displayed much less rapid growth than explants cultured in tumour extract.

3. The general cytology of the cultures has been described.

4. The origin of the pseudo-fibroblasts and clasmatocytes has been discussed.

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STUDIES ON MITOCHONDRIA

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INTRODUCTION.

The study of mitochondria is by no means new; ever since Altmann discovered these cyloplasmic inclusions in 1890 they have constantly attracted the attention of biologists. Later, when the special technique elaborated by Benda and Flemming made it possible to demonstrate these bodies consistently, they became the subject of much study by the investigators of the time, who attributed to them many revolutionary rôles (26, 9, 30, 13, 11), most of which, however, were discredited by later observers. Nevertheless, their work awoke and stimulated an interest in these intracellular bodies. Though the behaviour and function of mitochondria in cellular metabolism are still at the present time a matter of controversy among cytologists, an attempt is made in this paper to discuss and survey the recent literature dealing with the mitochondrial problem, with a view of helping to elucidate the nature of these cell-components.

OCCURRENCE AND MODE OF ORIGIN.

Mitochondria have been observed by various investigators in many types of cells ranging from protozoa to man, and from the angiosperm to myxomycetes. Guillermond (12) had even succeeded in detecting these bodies in certain algae and in diatoms. Their presence, however, in bacteria has been a matter of doubt until recently, when Alexieff (1) reported the presence of these bodies in some of the larger forms. Mitochondria have also been detected in non-nucleated blood corpuscles, but as they disappear with the appearance of haemoglobin. This phenomenon has given rise to the belief among a few investigators that the mitochondrial substance is converted into haemoglobin.

Recently, while observing mitochondrial behaviour during the life-cycle of Monocystis (24) it was noticed that at the commencement of the spore phase the mitochondria gradually decrease in numbers, and are totally absent within the mature spore. In general, it may be said, their existence is universal.

Most cytologists are of the opinion that mitochondria do not arise *de novo*, but always by the division of pre-existing ones (see figures 7, 8). Of late many investigators have observed this phenomenon within the living cell *in vitro*.

The theory of mitochondrial continuity is favoured by most workers, although Chambers (5), on the other hand, claims that he has actually detected mitochondria arising *de novo* in the protoplasm of certain protozoa. While observing the behaviour of these cell-components within living cells (23, 21), I was unable to confirm these observations of Chambers.

Some authors have gone so far as to suggest that mitochondria are secretory granules, which owe their origin directly to the Golgi network; and Nassonow (32), while studying the behaviour of the Golgi apparatus in the epididymis, describes how certain "granules," which he interprets as modified mitochondria, make their appearance in relationship with the network of the apparatus. Ludford, however, has shown that the mitochondria are quite distinct from these "granules"; and as mentioned in a previous paper (15), while observing the Golgi bodies of the ectodermal cells of *Hydra viridis*, I was unable to detect any topographical relationship between the Golgi apparatus and these granules.

ARRANGEMENT WITHIN THE CELL.

When examining sections of metazoon tissues, that have been treated by the usual mitochondrial technique, the mitochondria may be seen within the cell-protoplasm in the form of filaments, rod and spherical bodies. When the cells are observed individually it will be noticed, in the vast majority of cases, that the mitochondria occur in larger numbers around the nucleus and around the periphery of the cell. If, however, a section cut through the middle of the nucleus be examined, under an oil immersion lens, it will be observed that the mitochondria are arranged around the nucleus, apparently in intimate contact with the outer surface of the nuclear membrane (see figure 12). This interesting phenomenon may at first give the impression that these cell-components are actually traceable through the nuclear membrane; but a more detailed study shows quite clearly that these bodies lie in intimate relation only with the outer surface of the membrane, and do not occur within the nucleus. The few mitochondria, however, that come to lie in the cell-cytoplasm between the nucleus and periphery appear to be distributed without definite order throughout the

EXPLANATION OF PLATE 1.

All microphotographs were taken from material fixed in Flemming's solution without acetic, or else in a Champy's fixative. All sections were stained with Heidenhain's iron-haematoxylin, and occasionally counter-stained in eosin.

Figs. 1 to 4. Oblique and surface sections of Paramoecia demonstrating the orientation of mitochondria in the surface cytoplasm. Note how the mitochondria occur one behind the other, the long axes parallel with the long axis of the cell. Observe the arrangement of mitochondria around the meganuclei and periphery of the cells; also note their irregularity within the deeper parts of the organisms.

Fig. 1.

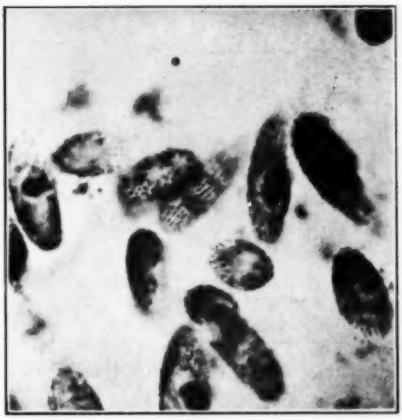

Fig. 2.

Fig. 3.

Fig. 4.

PLATE 1.

protoplasm. Recently, many authors, when discussing the arrangement of these bodies within the cell, have drawn attention to these various aggregations of mitochondria. In previous papers (17, 21) I have pointed out that these dense accumulations of mitochondria (such as are seen in many cells) may possibly be a surface tension phenomenon, for Mayer, Rathery, and Schaeffer (29) have recently contended that mitochondria are largely composed of phosphatides which contain fatty acid radicles; and moreover it is known that phosphatides, like other fatty substances, accumulate at the phase boundary of the cytoplasm, according to the Gibbs-Thomson law, owing to their capacity to reduce surface tension. It is therefore possible that the aggregations of mitochondria at the nuclear-cytoplasm interface may be related to their phosphatidal components. Such a conclusion would also help to explain the frequent occurrence of mitochondria around the vacuolar membranes and the peripheral region of many cells.

When studying the arrangement of these intracellular inclusions within the protozoan cell, this phenomenon is still more marked. During a previous investigation the arrangement of mitochondria in Nyctotherus and Paramoecium was studied in great detail (22). Similarly, large granular masses of mitochondria were observed around the outer surface of the meganuclei; but, while examining the surface sections of these organisms it was further observed that the rod-like mitochondria present in the surface cytoplasm exhibit a striking orientation (see figures 1, 2, 3, 4); and at the same time a closer observation revealed the mitochondria in greater abundance in this region of the infusorian than in the centre. A separate examination of Nyctotherus and Paramoecium shows a marked difference in the arrangement of orientation of the mitochondria in the surface protoplasm of these two organisms.

If, for example, a surface section of Paramoecium be observed, it will be seen that the rod-like mitochondria are arranged one behind the other in rows, each single mitochondrium having its long axis parallel to the long axis of the infusorian (see figures 1, 2, 3, 4); while, on the other hand, an examination of the orientation of mitochondria in Nyctotherus reveals these rod-like bodies arranged one behind the other in rows, each mitochondrium having its long axis more or less transverse to the long axis of the cell.

A more detailed study of the surface sections of both these organisms reveals the presence of myoneme threads, which show up quite clearly after the cell has been treated with ordinary mitochondrical fixatives (see figure 10). These myoneme threads run parallel to and beneath the rows of cilia, and according to Minchin (31) are a characteristic feature of the ciliate infusoria. These myoneme threads, which run in rows longitudinal and parallel to the axis of the cell, cause the surface cytoplasm to be thrown into folds or corrugations. As it has already been pointed out that the surface accumulations of mitochondria in cells may be interpreted in the light of their phosphatide composition, the same theory may be brought forward to explain how they come to lie

round the inner surface of the cell-membrane of the protozoan cell: the mitochondria would naturally collect in the corrugations between the intervening myoneme threads, which would thereby cause their segregation into distinct longitudinal rows.

Studies have demonstrated that in different infusoria the number and proximity of these myoneme threads varies. In Paramoecium, for example, they are so closely adjacent to one another that the mitochondria are forced to lie end on end, thus giving rise to the longitudinal orientation, as is shown by the micro-photographs on plate 1.

In Nyctotherus, on the contrary the distance between each myoneme thread is greater, thereby allowing the rod-like mitochondria to lie side by side in a position transverse to the axis of the cell. Thus it is suggested that the difference in the orientation of the mitochondria may be due to the differences in the proximity of these myoneme threads to one another.

QUANTITATIVE CHANGES.

The statement has frequently been made that the number of mitochondria is approximately constant within similar cells of the same species. Wakelin Barratt (3), for example, states that in the liver of the rabbit "the number of chondriosomes (mitochondria) present in a single cell is approximately 70, the variations met with not appearing to be considerable."

In a previous paper (23) I have estimated the number of mitochondria within living cells, and found that the numbers of these bodies in the cells observed varied greatly. In the series counted there was certainly no indication of a constancy in the number of mitochondria.

Of late, methods of counting these cell-components in unit areas have been elaborated by several investigators (37, 8). It is quite evident that the mitochondrial content cannot be used as a basis of classification.

Quantitative changes in the form of a diminution in the number of mitochondria are apparently met with in pathological conditions, and according to Cowdry (6) an increase above the normal is rare. However, it is of interest to note that Homans (14) reports a marked increase in the number of mitochondria in the islets of Langerhans during diabetes, while Policard (34) has

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EXPLANATION OF PLATE 2.

All microphotographs were taken from material fixed in an osmo-chromic solution stained with Heidenhain's iron-haematoxylin.

Fig. 5. Transverse section through the intestine of an adult land planarian. Note the highly vacuolated protoplasm of the phagocytic cell: many food vacuoles may be seen. Note the mitochondria embedded in the food globules within the digestive vacuoles.

Fig. 6. Longitudinal section of a binucleate opalina, showing the rod-shaped mitochondria lying in close connection with the vegetative granules.

Figs. 7 and 8. The same. Many mitochondria may be observed underlying a transverse binary fission. Note in fig. 7 the large mitochondrium near nucleus undergoing division.

Fig. 5.

Fig. 6.

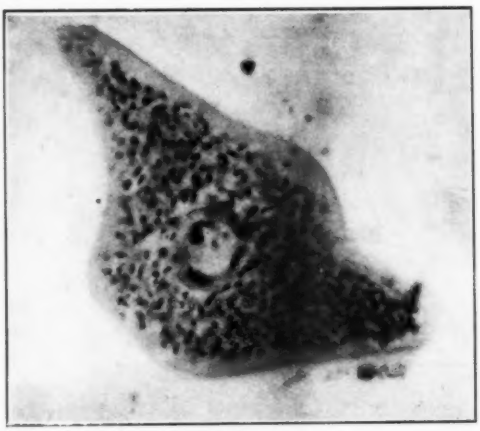

Fig. 7.

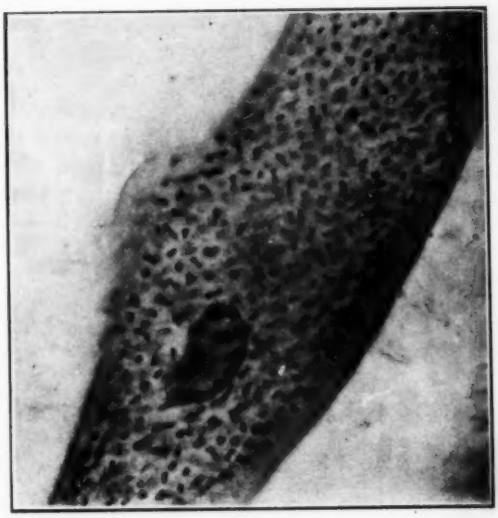

Fig. 8.

PLATE 2.

succeeded in increasing the number of these bodies in the kidney following the administration of phloridzin. Recently, while studying the qualitative and quantitative changes of mitochondria in the parenchymatous cells of several types of regenerating landplanarians, an enormous increase in the number of mitochondria in the regenerating tissues was observed. The cells were so densely packed with mitochondria that it was quite impossible to estimate their numbers in unit areas (see figures 13, 14, 16), while on the other hand the number of mitochondria in the cells of the control organisms roughly varied between 16–30 (see figure 15).

The significance of this marked increase is doubtful: whether the increased number of mitochondria in the regenerating tissues be due to cellular injury, or whether it may be assumed that these inclusions play an active rôle in cellular differentiation, it is difficult to say. It is significant, however, that mitochondria are sensitive to cell injury, and investigators have shown that in many cases injury to the cell produces a change and increase in the number of these bodies. In glands, for example, they respond to injury by loss of the filamentous mitochondria; these break up into numerous, small, rod-like bodies, which in their turn rapidly multiply by a transverse binary fission.

On the other hand, it has been estimated that embryonic cells contain approximately more mitochondria per unit volume of cytoplasm than adult cells, and, moreover, that they disappear as the tissue become more senile. Furthermore, Cowdry (6) states that "as development progresses distinctive differences in the amount of mitochondria arise with the specialization of tissues." It is, however, agreed upon by most cytologists that an increase in the number of mitochondria is suggestive of a heightened activity, while a decrease indicates a depression of functional activity.

FUNCTION AND DISCUSSION.

That mitochondria play an important rôle in cellular metabolism was first put forward by Lewitski (27), Guillermond (13), and Forenbacker (10), who suggested that starch and other plastids produced in plant cells owe their origin indirectly to these cell-components. Later, other observers demonstrated that plastids are in reality transformed mitochondria, whose function is the production of starch, chlorophyll, anthocyanins, fat, etc., according to whether they become amyloplasts, chloroplasts, chromoplasts, elaioplasts, or other such cytoplasmic structures. Later Cowdry (7) formulated the theory that these structures are originally formed within the mitochondria, which later enlarge to form the body of the plastid, while other writers suggested that mitochondria have the power to select certain material from the surrounding protoplasm of the cell, and build it up into various vegetative products.

These observations led to the development of the "electosome" theory of

Regaud (35), according to which mitochondria are "electosomes" which have a specific selective action upon the surrounding protoplasm, and are centres of specific chemical elaboration and accumulation.

Recently, while studying the behaviour of the cytoplasmic inclusions within a common endo-parasitic protozoan (binucleate Opalina, 18), I found that in close connection with each single mitochondrium there may develop a large granule (see figure 6). From this it appears that in Opalina a metabolism, involving the visible condensation of vegetative material, is occurring at the surface of the mitochondria. These observations seem to lend strong support to the speculation that mitochondria are the loci of protein, and possibly of general protoplasmic synthesis within the body of the living cell.

While studying the behaviour of mitochondria throughout the life history of Monocystis it was observed that these cell-components gradually decrease in number at the commencement of the spore phase, and are totally absent when the spore is mature, but reappear in the free, active sporozoite at a time when chemical resynthesis occurs (24). This evidence again seems to suggest that mitochondria are correlated with cell-synthesis. Further light is thrown upon the mechanism of this synthetic activity by the recent development of an enzymatic conception of mitochondrial behaviour, a development mainly due to the work of Marston and Robertson. Marston (28) concludes on the basis of their reactions to azin dyes that mitochondria contain proteolytic enzymes; and, considering the rapidity with which synthetic processes take place in living tissues, he develops the conception of mitochondria as the site of enzymatic synthesis in the cell, the "water-poor" phases which exist at the surface of the lipoidal constituents of the mitochondria providing suitable conditions of synthetic activity.

While discussing this phenomenon it is of interest to note the recent conclusions of de Nouy and Cowdry (33), who contend that mitochondrial substance is arranged in such a way as to give a surface film of maximum extent with a minimum amount of material: such a local concentration would furnish an

EXPLANATION OF PLATE 3.

All microphotographs were taken after the material had been treated in Flemming's solution without acetic, and stained in Heidenhain's iron-haematoxylin, except fig. 9, which has been counter-stained in eosin.

Fig. 9. Section through the pancreas showing the darkly staining zymogen granules in the aveolar cells, which have arisen from the filamentous mitochondria. Observe the secreted granules in the acini ducts.

Fig. 10. Oblique surface section through an infusorian showing the longitudinal myoneme threads running in rows parallel to the axis of the cell.

Fig. 11. Portion of a transverse section of a grain of maize, fixed on the fifth day after germination, showing a portion of the scutellar epithelium. Note the deeply staining nuclei of the epithelial cells. Observe the secreted mitochondria lying in the crushed wall of the endosperm.

Fig. 12. Section cut through the parenchymatous tissue of an adult land planarian, showing the dense aggregation of mitochondria around the outer surface of the nucleus.

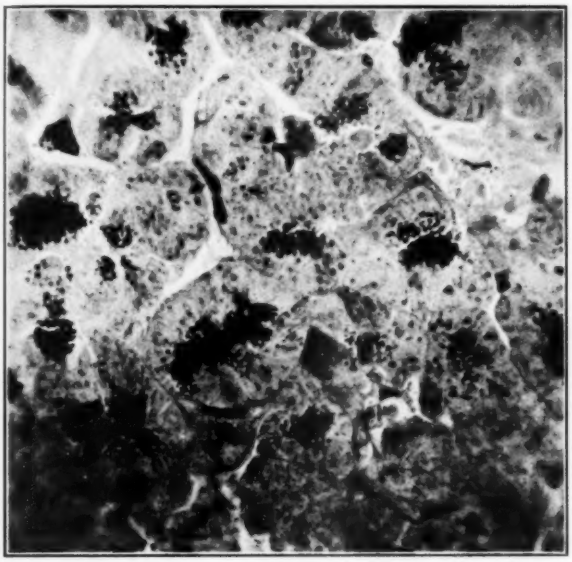

Fig. 9.

Fig. 10.

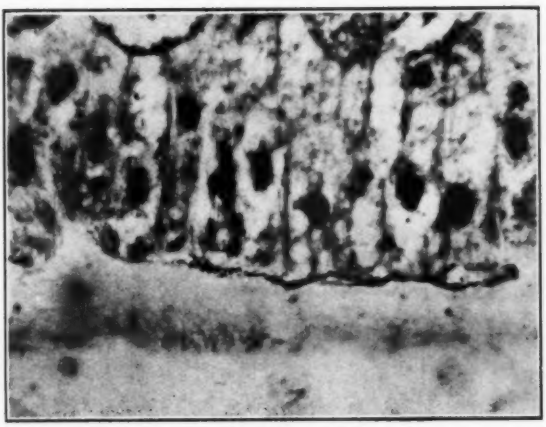

Fig. 11.

Fig. 12.

PLATE 3.

explanation of the facility with which enzymatic synthesis takes place at the surface of these cell-components. Moreover, the behaviour of mitochondria appears to illustrate the capacity of enzymes to reverse their activity in agreement with conditions, i.e., synthesis or hydrolysis, according to the concentration at the substrate; and it has likewise been shown that mitochondria are also concerned with the latter phenomenon.

Regaud and Mawas (35) have demonstrated that the zymogen granules which are secreted from the salivary glands arise from the filamentous mitochondria; and in the case of the pancreas, observations have shown that these bodies break down into zymogen grains, and eventually pass into the acini (see figure 9), thus indicating that mitochondria are the source of the tryptic secretion (16, 21).

An increase in the permeability of the plasma-membrane, which is thought to occur (Rogers, 36) during secretion might facilitate the escape of these granules from the cell.

In the process of intracellular digestion in several types of planaria and protozoa (20, 19), it has been shown that the mitochondria become included in the food vacuoles of these organisms, suggesting that subsequently the mitochondria give rise to the enzymes concerned in digestive activity. Observations have also been made upon the relation of mitochondria to the process of intracellular digestion within the living protozoan cell (21, 23); the engulfed food at first circulates in the protoplasm, at the same time coming in contact with the cell mitochondria, which then adhere to it. Subsequently a vacuole forms around the food particle, together with its associated mitochondria, after which the latter pass into solution and disappear, and the engulfed foreign particle undergoes disintegration.

Thus it is again concluded that mitochondria are concerned with the production of the enzymes which bring about the hydrolysis of the engulfed food material.

Causey (4), while investigating mitochondrial behaviour in *Entamoeba gingivalis*, also detected these bodies lying within the interior of the food vacuoles by vitally staining fresh material with Janus green B. As the latter is a specific stain for mitochondria alone (7), there can be very little doubt concerning the nature of these bodies. While dealing with the process of intracellular digestion, Rogers (36) describes how certain granules (which may apparently be interpreted as mitochondria) are seen to aggregate in the protoplasm surrounding the membranes of the digestive vacuoles, which they finally enter. This writer further suggests that these cell-components are the carriers of the tryptic enzymes in the inactive or zymogenic state, and, moreover, that the acid which is found to be present within the food vacuoles of these organisms plays the part of an activator, thereby changing the inactive zymogen into the active ferment.

Similarly, while observing mitochondrial behaviour during the germination of cereals, it has been shown that mitochondria are associated intimately with the hydrolysis of the starch grains (25), being secreted in large numbers from the epithelial cells of the scutellum into the adjacent starch-containing cells of the endosperm (see figure 11). These secreted bodies then aggregate round the starch grains leading to their corrosion; and as the latter proceeds the associated mitochondria disappear.

This behaviour of mitochondria lends a considerable support to the enzymatic conception of mitochondrial activity, it being supposed that the starch-splitting enzyme is located within them or at their surface, and is liberated therefrom when these bodies have reached the surface of the starch grains.

CONCLUSIONS.

The recent literature dealing with the mitochondrial problem has been reviewed, and the apparent function and behaviour of mitochondria within the animal and plant cell have been discussed. The arrangement of these bodies around the periphery and nucleus of the cell appears to be a surface tension phenomenon dependent upon their phosphatide components; and the differences in the orientation of mitochondria in the surface cytoplasm of infusorians may be correlated with the presence and proximity of the myoneme threads.

The general behaviour of these cell-components during cellular metabolism appears to lend considerable support to an enzymatic conception of mitochondrial activity.

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EXPLANATION OF PLATE 4.

All microphotographs were taken from material fixed in Bensley's solution stained in iron-haematoxylin.

Figs. 13, 14, 16. Sections of parenchymatous tissues of regenerating land planaria showing the enormous increase in the number of cell-mitochondria.

Fig. 15. Section through the parenchyma of a normal land planarian showing the darkly staining mitochondria. The highly staining granules seen in the anterior cells are vegetative.

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TYPE DIFFERENCES AMONGST STAPHYLOCOCCAL BACTERIOPHAGES

by

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The work of Bail (1923) and his school has established the possibility of differentiating bacteriophages active against the intestinal bacilli into well-defined types. His primary differentiation was based on the size of the plaques produced on agar, but this was soon extended by the study of the power of phages to produce specifically resistant variants. It is very easy to obtain from suitable sources phages active against *B. dysenteriae* Shiga. Some of these will be found to produce large plaques on agar, some small. By incubating broth with some of the material at the centre of an isolated plaque, "pure" phages may be obtained, giving plaques of fairly constant diameter. If a Shiga culture is now lysed by two such pure phages, one "large" and the other "small," resistant cultures may be obtained and purified by successive replating. It is then found that the culture resistant to the large plaque phage remains sensitive to that producing small plaques, and *vice versa*. The two phages thus show a clear-cut functional difference as well as a difference in plaque size. This method of study by "cross-resistance" experiments may be applied to any number of phages lysing a single strain. Two phages lysing the same strain are of the same type when any culture rendered resistant to one is also resistant to the other. Amongst Shiga phages Bail and Watanabe (1923) found that about eight types could be differentiated in this way. In the Salmonella group one of us found the same general rules to hold (Burnet, 1927), and Hadley (1928) has made similar observations on paratyphoid phages.

No comparable observations on staphylococcal bacteriophages appear to have been published, probably because of the great difficulty in obtaining active staphylococcal phages with any regularity.

Previous work (Burnet, 1928) had shown that with a sufficiently extensive series of phages striking relationships between the smooth-rough interchange and phage reactions could be observed in the Salmonella group, and it was hoped that by following similar lines it would be possible to determine whether anything equivalent to "rough" change occurred in the staphylococci.

A large number of filtrates were prepared from staphylococcal lesions of various sorts, but active phages were very rarely encountered. Four were studied, but no strain of pyogenic staphylococcus was found sensitive to more than two of the phages. In the course of a search for further phages a white staphylococcus was isolated from guinea-pig faeces. This organism was lysed with filtrates made from the faeces of rabbits, rats, and guinea-pigs, and several phages were isolated. These form the main subject of the present communication.

Characteristics of the Sensitive Staphylococcal Strain.

The staphylococcus SF grew well on ordinary media in typical white opaque colonies of normal consistency and smooth surface. Microscopically the cocci were regularly Gram positive, round, and uniform in size; in broth diplococci were numerous, and an occasional chain of 3 or 4 units could be seen, but for the most part the organisms were present as single cocci or in small, irregular clumps.

There was no trace of haemolysis on blood agar, and the organism did not clot citrated plasma or liquefy gelatine. It was non-pathogenic to mice and rabbits.

It produced acid in lactose, salicin, mannite, and raffinose, but not in inulin. Peroxidase and catalase reactions were strongly positive, and the strain did not grow anaerobically. Except for its power to ferment mannite the organism is therefore a typical non-pyogenic *Staphylococcus albus*.

Description of the Phages Used.

The phages were obtained from rodent faeces. The faeces, a number of pellets from several different animals of the same species, were added to 20 ccs. of broth. After incubation for two hours the pellets were broken up with a sterile glass rod and again incubated for two hours. The culture was then filtered through paper, and finally through a Seitz filter. The primary filtrates were tested on the strain SF by adding a loopful of the filtrate to an agar surface previously spread with a broth culture of the organism. If this failed to show any phage action, some of the primary filtrate was added to a broth culture of SF, and the culture filtered after incubation overnight. When any evidence of phage appeared, suitable dilutions to give isolated plaques were spread on agar with the sensitive strain. The stock phages were then derived from single plaques. Five recognizably different phage types were obtained whose general characteristics may be given.

(A) A weak phage from rabbit faeces, showing very transient or no clearing of a broth culture. After incubation overnight a filtrate gave about 400 million plaques per cc. The plaques were of medium size (1–1·5 mm. in diameter on

1·5% agar), and showed a characteristic arrangement of tiny resistant colonies in the centre of the plaque.

(B) An active phage obtained from guinea-pig faeces, that showed good clearing of a broth culture, followed at a variable interval by secondary growth. The usual titre was about 300 million particles per cc. of filtrate. The plaques were small (up to 1 mm. in diameter), sharp edged, with clean base.

(C) This phage showed a good deal of variability in some of its characteristics: as isolated from rat faeces it produced larger plaques than is usual with staphylococcal phages (up to 2·2 mm. in diameter). The plaques showed always a central mass of opaque resistant growth with a clear periphery. Titres of broth culture filtrates varied greatly without obvious reason from 1 million to 300 million per cc. After several transfers this phage showed when plated a second type of plaque of the same large size, but lacking the central growth (see figure 1). A phage derived from one of these plaques consistently gave rise to the same large, clear plaques, but in its behaviour toward the various resistant strains to be described later showed no changes from the original phage.[1] Another phage similar to the original (C) was derived from guinea-pig faeces.

(D) This was the most active phage obtained. Cleared broth cultures remained limpid indefinitely at room temperature, but at 37° a slight turbidity developed after some days, and became gradually more marked. Small, clear-cut plaques were produced on agar (figure 1), and there was a complete absence of resistant colonies on the clear areas. The phage was derived from rat faeces.

(E) Another active phage from rat faeces. Lysed cultures usually remained clear at room temperature, but resistant cultures were more readily produced than with D. It gave small, clear-cut plaques on agar up to 1 mm. in diameter. Unlike all the other phages this showed an inhibitory action against some pyogenic staphylococci on agar. Efforts to obtain multiplication at their expense, however, were unsuccessful.

Resistant Cultures.

The usual methods were employed in an attempt to obtain cultures resistant to each of these phage types. No difficulty was experienced in obtaining them with phages A, B, and C, either from the secondary culture in broth or from the colonies appearing on cleared areas of agar cultures. Phage E usually gave only abortive or nibbled colonies after lysis in broth, but by prolonged incubation resistant forms of considerable persistence were obtained. No true resistant

[1] It is worth mentioning that the plaques of this phage showed very extensive further spread on prolonging incubation. On a plate of double thickness nutrient agar 6 plaques at 18 hours showed diameters of 1·84, 1·72, 1·44, 1·54, 1·18, and 1·16 mm. respectively. The same plaques after incubation for four more days had diameters of 4·72, 4·40, 4·63, 3·76, 4·07, and 3·00 mm. This was the limit of complete clearing, but beyond this was a definite halo in most cases having a diameter of about a centimetre. It will be remembered that d'Herelle claimed in his controversy with Twort that once definitely formed a plaque never increased its diameter on further incubation.

cultures have been as yet obtained with phage D. The moderate turbidity present after prolonged incubation of a broth culture of SF lysed by phage D is apparently always due to the presence of temporarily resistant forms that grow on agar only as "flatterformen" or nibbled colonies.

The resistant colonies eventually obtained after lysis by any given phage were rarely entirely uniform in appearance, and several types were always tested. One corresponding to each phage was chosen as a typical resistant form and used for further study. The reactions of such typical resistant forms towards the five phages were as tabulated.

TABLE 1.

Reactions of Resistant Cultures toward Phage.

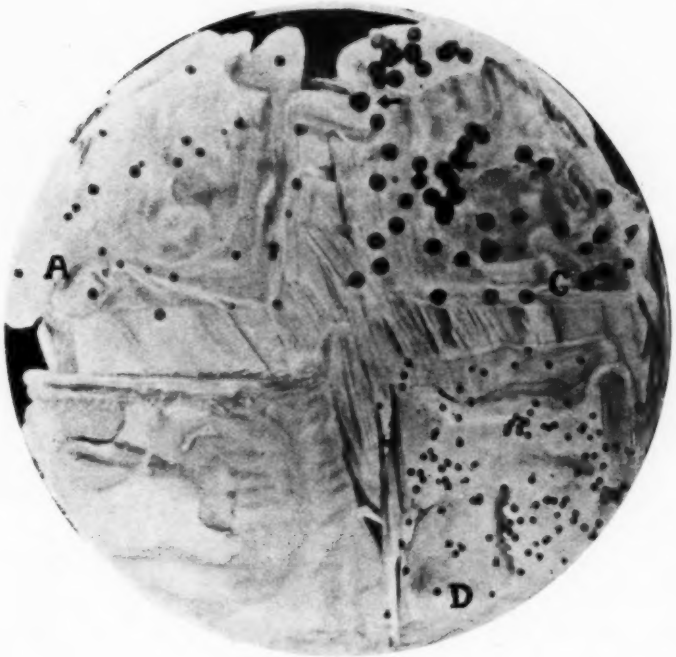
Strain.	A	B	C	D	E
S.F. original	†††	†††	†††	†††	†††
S.F./A	—	†††	†††	†††	†††
S.F./B	†††	—	—	†††	†††
S.F./C	†††	†††	—	†††	†††
S.F./E	†††	†	—	†††	—

All five phages show clear individuality, but there seems to be some relationship between B, C, and E, since strains resistant to B and to E were always also resistant to C. Only one strain resistant to E was obtained, but numerous cultures resistant to B or C were obtained and examined. Three types resistant to B could be obtained, differentiable by the colour of their colonies. The most frequent was opaque white, identical in appearance with the parent form; the second was colourless and translucent; the third a frank aureus. All showed the same reactions to phage.

With phage C three types could also be distinguished: one opaque, whose reactions are given above as the typical form. The other two correspond in morphology and in phage reactions with the two white types of SF/B.

Heat Resistance of the Phage Types.

The resistance of the first four phages to heat was tested by heating a few drops of filtrate in a sealed capillary tube completely immersed for half an hour in a waterbath at the required temperature. The contents of the tube were then blown out on to an agar plate previously spread with a broth culture of SF1, and the plate incubated at 33°. As others have found, there are liable to be inexplicable differences in the results of such experiments, and Table 2 shows a few discrepancies, but its general appearance will indicate that there are very characteristic differences in heat resistance of these four phages. At least two different "generations" of each phage were used, and the experiments were repeated for the range over which complete inactivation took place.



DESCRIPTION OF PLATE.

Discrete plaques produced by phages A, C, and D on the Staphylococcus SF. Phage C shows in addition to the original type two plaques, indicated by arrows, of the more active type described in the text.

TABLE 2.

Heat Resistance of Phages A, B, C, and D.

Activity of filtrates on S.F. after heating 30 minutes at the temperatures shown.

Temp.	Phage A.	Phage B	Phage C.	Phage D.
72·5° C. ..	—, —	—, —		
70° ..	—, —	—, —		
67·5° ..	—, †, †, †, —	†, —, —, —, —, †,	—, —	—, —
65° ..	†, †, †, †	—, —, —, —, —, †	—, —, —, —	—, —
62·5° ..	†, †	†, —, †, †	—, —, —, —	—, —
60° ..	††, ††	†, †††	—, †, †, †, †	—, —, —, —, —
57·5° ..			†††, †††, †††	—, —, —, —,
55° ..				†, †, †, —

††† confluent lysis; †† semi-confluent lysis; † discrete plaques; —, no lysis.

Phage A is the most resistant, its power of producing plaques on agar being destroyed at about 67·5°, while phage D is extremely sensitive to heat. It is almost completely inactivated, even at 55°, despite the fact that it is the strongest of the four phages under consideration. Phage C is regularly inactivated just above 60°. Results with B have been irregular; there are usually isolated plaques after heating at 62·5°, and occasionally a few persist even at 67·5°.

All these results refer to filtrates from the original lysed cultures containing for A, B, and D between 200 and 500 million particles per cc. The filtrates of C were weaker, those used having about 1 and 2 million particles respectively. If the phages were diluted in saline or phosphate solution to about 1%, they were all completely inactivated at 55°, with the exception of C, which gave a few plaques. They all survived this temperature, with considerable numerical weakening when similarly diluted in broth.

It is fully recognized that loss of power to produce plaques on agar may not signify that the phage particles are incapable of again giving rise to phage on passage in broth, but the present experiments are only concerned to demonstrate the individuality of the four phages as regards this particular function.

Characteristics of Resistant Cultures apart from their Reactions to Phage.

Colour.

Most of the resistant cultures showed the same opaque chalky-white appearance on agar as the original strain. Amongst the resistant colonies appearing after lysis by phage B, however, two other types appeared. One showed no pigmentation, but was much less opaque than the typical forms, almost translucent. It was nonlysogenic, and showed the phage reactions given by typical SF/B variants, but on being kept on agar for some weeks gave rise to papillae, some of which were of the chalky-white type, others frankly aureus. Either in this way, or directly after lysis of SF by phage B, the second (aureus) type of SF/B could be obtained. Variants of this type were obtained in four separate experiments, and always showed the characteristic phage sensitivity, so that

there can be no question of accidental contamination having been responsible for their appearance. The pigment seemed to be identical with that of ordinary pyogenic strains of *S. aureus*, and like this did not develop well until the culture had been left a few days at room temperature after incubation.

Biochemical Activities.

None of the variants, including the aureus strains, were haemolytic on blood agar, and none clotted citrated plasma. Their sugar reactions after seven days' incubation were identical with those of the original strain, although there were differences in the rapidity with which acid was formed. All showed strong peroxidase and catalase reactions, and SF/B (aureus), like the parent strain, failed to grow anaerobically. In no respect other than colour, therefore, does the aureus variant approach the characters of the pyogenic staphylococci, and like the parent strain it is totally nonpathogenic for mice and rabbits.

Agglutination Reactions.

All the cultures gave stable emulsions in normal saline, and were agglutinated by specific sera in granular fashion. The agglutinability of emulsions was not affected by boiling for twenty minutes. Sera were made by immunizing rabbits with the four strains, SF, SF/B (aureus type), SF/A, and SF/C, the first two in the form of killed cultures. The sera against SF/A and SF/C were made later, when the complete nonpathogenicity of the strains had been established, and by the use of large doses of living cocci higher titres were obtained. The sera were studied by the ordinary methods of agglutinin absorption, and the data, in the form of limiting titres of microscopically visible agglutination, are combined in Table 3.

TABLE 3.

Agglutination Reactions of Staphylococcus SF and its Resistant Strains.

Serum.	Absorbed by.	Agglutinating Emusion.			
		SF	SF/A	SF/B	SF/C
SF	nil	640	640	320	320
	SF/A	400	<50	200	200
	SF/B	320	320	<40	80
	SF/C	<50	<50	<50	<50
SF/A	nil	1280	2560	640	1280
	SF	<100	<100	<100	<100
	SF/B	800	1600	<100	1600
SF/B	nil	1280	1280	1280	1280
	SF	<40	<40	<40	<40
	SF/A	1280	<40	1280	640
SF/C	nil	5120	5120	2560	5120
	SF	<100	<100	<100	<100
	SF/A	3800	<120	1920	3800
	SF/B	400	800	<100	1920

The relationships between the parent strain and the two resistant forms, SF/A and SF/B, are clearly shown in the table. It will be seen that although all the agglutinins of serum SF/A are exhausted by SF, when serum SF is absorbed with SF/A it still remains capable of agglutinating SF and SF/B. The strain SF/A therefore lacks an antigenic component present in SF and in SF/B.

Similarly the serum SF/B is completely absorbed by SF, while SF serum absorbed by SF/B contains residual agglutinins acting on SF and SF/A. SF/B therefore also lacks a distinctive antigenic component present in SF and SF/A.

All the findings in the table relevant to these three strains are thus adequately co-ordinated if we regard SF as containing three antigenic components, α, β, γ; SF/A will then be represented by $\beta\gamma$, and SF/B as $\alpha\gamma$. Strain SF/C is serologically almost identical with SF. Both strains can exhaust the opposite serum of all agglutinins, and the only clear difference in behaviour is found when their sera are absorbed by SF/B. In each case the homologous strain is then agglutinated to a higher titre than the heterologous one. The only explanation that can be offered is that the component unabsorbed by SF/B, i.e., β differs slightly in the two strains.

The strain SF/E as first isolated appeared to be a true resistant form, and it was used along with the others for serological work. Its reactions were rather confusing, resembling on the whole those of SF/C. It was found, however, that the culture was undergoing reversion to the normal sensitive type, and its agglutination reactions have therefore been omitted from the table.

DISCUSSION.

The results described in this paper fall into two groups, one bearing on the individuality of phages active against the same staphylococcal strain, the other on the antigenic and other changes in the bacteria that are associated with resistance to the different phage types. These may be discussed separately.

(1) Early in his work d'Herelle insisted that definite individuality could be recognized amongst the phages active against and propagated on a single bacterial strain. He used the activity (or virulence) of the phage in lysing the substrate strain and its range of action on heterologous species as his chief criteria for recognizing an individuality that persisted through successive generations. Bail (1923) recognized that differences in the size of plaques on agar did not represent, as d'Herelle thought, mere differences in virulence, but could be used to characterize phage types. Following up the significance of these differences in size, he found that the resistant colonies appearing after lysis by a large plaque phage were still sensitive to lysis by a small plaque phage, and *vice versa*. This led to a more reliable method of differentiation than simple inspection of plaques, and with his collaborators, particularly Wantanabe (1923),

he was able to differentiate eight phage types active against *B. dysenteriae* Shiga by the use of "cross-resistance" tests. Outside Bail's school the method has not been widely used, but one of us has found the same general rules applying to phages lysing certain Salmonellas, and Hadley has made some studies on large and small plaque phages active on the paratyphoid bacilli.

Phages active on a single strain can be further differentiated by the use of antibacteriophage sera [Wantanabe (1923), Bruynoghe (1923), Muckenfuss (1928)], and sometimes by differences in their resistance to heat [Gratia (1923), Hadley (1928)].

Few authors seem to have worked with more than a single phage active against any one staphylococcal strain. The chief exceptions are Gratia and de Namur (1922), who described two phages both capable of lysing the one strain, a white staphylococcus. One phage, V, was confined in its action to this strain, the other, H, was capable of lysing practically all the staphylococci tested. Both were passed on this one strain for many generations without any loss of their specific characters. H remained polyvalent; V still acted on the one strain only. Specific antisera prepared against the two phages inhibited the homologous phage only. The two phages therefore maintain their individuality, despite the fact that they are propagated at the expense of the one strain. Gratia and de Namur note that this may be taken as evidence in favour of d'Herelle's theory, but consider that the facts can also be interpreted according to Bordet's view of the nature of phage.

The present work extends the findings of these authors, and shows that with one staphylococcal strain at least five distinct phage types can be propagated and retain their distinctive characters through several transfers. The points used for differentiation are (1) type of plaque on agar (2) nature of the resistance found in secondary cultures, and (3) heat resistance of the phages. The differences in heat resistance are particularly noteworthy, and show the impossibility of making any general statements about the thermal "death point" for staphylococcal phages. A point of interest is that the most sensitive phage D is a small plaque type. The relative heat sensitivity of the large plaque phages lysing intestinal bacilli, demonstrated by Gratia and Hadley, therefore does not apply to the staphylococcal phages.

The findings presented may be regarded from a general standpoint as confirming for another group of bacteria the persistent and genetically transmitted individuality of bacteriophage types that has been described for intestinal bacteria by d'Herelle, Bail, Flu, Schuurman, and others, and that has never been adequately interpreted by any opponent of the theory that bacteriophage is an autonomous living unit.

(2) The derivation of a strain of typical aureus colour from a saprophytic white staphylococcus is of some interest. Hucker (1925) found that amongst

the micrococci as a whole, pathogenic and non-pathogenic, colour was one of the most stable properties. There are a number of references in the older literature (see Eisenberg, 1914) to changes from white to aureus, and *vice versa*, amongst pyogenic staphylococci, and recently Bigger, Boland, and O'Meara (1927) have described a number of albus and other variants from pyogenic aureus strains. Apart from its colour the present variant showed no approach toward the specific characters of pyogenic strains. Its appearance may be related to the occurrence of chromogenic variants of other usually unpigmented bacteria, such as the phage-resistant derivatives of *B. proteus* described by Fejgin (1927).

The antigenic changes associated with resistance to phage are of more importance. It has been shown that the original strain can be regarded as antigenically a mosaic of at least three components, α, β, and γ. Associated with resistance to phage A there is a loss of component α, and with resistance to phage B the component β disappears. These findings were quite regular. As well as the stock strains used for immunization two other strains resistant to A that showed some differences in the morphology of their colonies proved to be equally lacking in component α. A large number of strains resistant to B were tested, aureus, opaque albus, and translucent strains derived from the interaction of SF with phage B, and both opaque and translucent strains produced by phage C, but at the same time resistant to B. All these failed to agglutinate with serum that had been absorbed by the aureus strain SF/B. Resistance to phages A and B therefore seems quite definitely associated with the absence of the respective components α and β.

The behaviour of phage C and its resistant forms is particularly interesting. Amongst the colonies appearing after lysis on agar from 20% to 50% show, after replating, sensitivity to phage B but resistance to C. Two were tested serologically, and showed the presence of the β component (i.e., they agglutinated to 1 : 160 with serum SF absorbed by SF/B). The remaining colonies (two types) were resistant to phage B as well as C, and on testing four serologically no β component was detected.

The strain SF/C (sensitive to phage B) is serologically almost identical with the parent strain. Both absorb all agglutinins from the heterologous serum, but there appears to be a significant difference when the sera are absorbed with SF/B, leaving only the β agglutinin. With such absorbed sera the homologous strain is agglutinated to a higher titre than the heterologous. This behaviour is not seen when the sera are absorbed by SF/A, and it must be concluded that while component α is the same in each there is some qualitative difference between the β component in the original strain and that in SF/C.

Apart from all the evidence that has been previously accumulated by one of us (Burnet, 1927, 1928), that phage sensitivity is primarily determined by the nature of the heat-stable agglutinogens of the bacteria, the above findings

in themselves are almost sufficient to substantiate this view. If a strain of SF posesses component α it is lysed by phage A, and if it lacks α it is resistant to the phage. We would interpret this to mean that phage A is specifically adsorbed to the same surface constituent as adsorbs agglutinin α, and similarly phage B and agglutinin β have the same point of attack. Phage C is also adsorbed to the same bacterial component as B, but it is more sensitive to slight alterations than the latter.

The results with the resistant strain SF/C are specially significant, as they afford a justification for the view that antigenic changes may occur without loss of the power of complete mutual absorption between parent strain and variant. In this case the two sera when absorbed of all but the component suspected from bacteriophage reactions to be different, showed distinct differences in their agglutinating powers for the two strains. In similar experiments with *B. sanguinarium* it was shown (Burnet, 1929) that no difference between the variant resistant to phage and the parent strain could be detected by cross-absorption methods, although the changed power of absorbing phage made it almost certain that some antigenic change had occurred. The method used with SF/C could not be applied there, but it was assumed that the same sort of antigenic change occurred, as has been actually demonstrated in the case of SF/C.

It may be pointed out here that the three phages whose resistant forms retained their specific characters regularly are weaker than the two, D and E, that produced only resistant forms of very slight or limited persistence. There is a definite suggestion that the development of resistance when it does occur with extremely active phages is a more active process than the selective action on spontaneously occurring variants which is responsible for the appearance of resistant cultures with weaker phages. But this in no way invalidates the conclusions drawn from the behaviour of the latter.

It may be concluded that with this particular organism the development of persisting resistance to phage is associated with demonstrable antigenic changes of two types. The simplest is the loss of a unit from the antigenic mosaic, the particular unit lost determining toward which phage the strain will be found resistant. The second type involves some alteration within one unit of the mosaic by which it becomes unsuited to adsorb the phage involved, and shows slight differences in serological behaviour, although still capable of absorbing all agglutinins corresponding to the modified or unmodified antigenic unit.

These findings might not, of course, apply to any other organisms than this particular type of staphylococcus, but, considered with the results previously reported for the Salmonellas, they constitute what is probably the most direct evidence yet put forward of the dependence between heat-stable antigenic structure (the only type present in staphylococci) and sensitivity to lysis by bacteriophage.

SUMMARY.

1. Five bacteriophages, active against the same strain of *Staphylococcus albus*, showed constant and distinctive differences through several generations in regard to:

(a) The size and nature of the plaques produced;

(b) Their heat resistance;

(c) The type of resistant culture produced.

2. Amongst the variants resistant to one type of phage a well-pigmented aureus strain was obtained.

3. All the resistant cultures showed some alteration in antigenic structure as judged by agglutinin absorption methods. Two types could be distinguished:

(a) Loss of a definite antigenic unit;

(b) Alteration within such a unit.

4. These results are discussed in support of the hypothesis that bacterial susceptibility to phage lysis depends primarily on the nature of the "heat-stable" agglutinogens of the organism.

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A COLORIMETRIC METHOD OF ESTIMATING GUANINE AND ITS APPLICATION TO THE DETERMINATION OF NUCLEO-CYTOPLASMIC RATIOS

(FIRST COMMUNICATION)

by

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INTRODUCTION.

The estimation of nucleo-cytoplasmic ratios by direct microscopic measurement of nuclear volumes involves many sources of error and uncertainty, which have been fully discussed by Conklin (3). On the other hand, the constituents of the nucleus which are stained by haematoxylin are, as A. P. Mathews has shown, nucleic acids (11). The identification of this distinctly chromosomal constituent opens up the possibility of measuring nucleo-cytoplasmic ratios in terms of the ratio of nucleic acid to the distinctively cytoplasmic constituent, protein.

The attempt to estimate nucleo-cytoplasmic ratios by chemical means is rendered difficult by the fact that nucleic acids themselves are not readily isolated without incurring important quantitative losses. Failing isolation of the nucleic acids themselves two alternatives remain. The nucleic acids may be estimated from the phosphoric acid which they yield when hydrolyzed, or from the purines or pyrimidines which are also set free on hydrolysis.

The estimation of the nucleic acid content of tissues in terms of phosphoric acid is complicated by the fact that many substances in tissues will yield phosphoric acid on hydrolysis. Such an estimation, therefore, ultimately depends upon the preliminary removal of other substances which may yield phosphoric acid, or the partial separation of the nucleic acids themselves from the other constituents of the tissues.

It has been suggested by LeBreton and Schaeffer (8) that the estimation of amino-purines would afford a reliable estimate of the mass of nuclear (chromatin) material in the developing organism. This method possesses the very important advantage that the purines of extra-nuclear origin are, in animal tissues at all events, oxy-purines, and not amino-purines.

Since it proved impossible to separate amino-purines from oxy-purines by direct precipitation, and the amino-nitrogen in amino-purines is not liberated quantitatively by nitrous acid (16), LeBreton and Schaeffer have now abandoned this promising method except for the early stages of embryonic development, during which oxy-purines do not occur in animal tissues. We are thus thrown back once more upon the difficult and uncertain method of directly estimating phosphoric acid of nuclear origin.

If either of the amino-purines (adenine or guanine) could be quantitatively estimated it would furnish as good a measure of nuclear (chromatin) mass as the two taken together, since they occur in nucleic acids in equimolecular proportions. It follows that a practicable method of estimating guanine would afford a satisfactory substitute for the method which LeBreton and Schaeffer have been forced to abandon, with the great advantage that purines of extra-nuclear origin would not interfere with the accuracy of the estimations.

A possible method of estimating guanine is indicated by the fact, discovered by Strecker (14), that guanine, treated with hydrochloric acid and potassium chlorate, yields guanidine. The results of Strecker do not indicate the extent of the yield of guanidine which is obtained under these conditions, but as it is obvious from inspection that no other purine would yield this product it is clear that the reaction must be highly specific for guanine, and therefore important to investigate further from the point of view of its utilization for the quantitative estimation of this purine.

The estimation of guanidine presents no difficulties since the discovery by Tiegs (15) and Marston (9) of a reagent which yields a specific colouration with this substance.

The reaction with hydrochloric acid and potassium chlorate proved difficult to control, for reasons which will be fully discussed in a subsequent communication.[1] Many oxidizing reagents were employed, but eventually potassium permanganate in hot hydrochloric acid solution was found to be the most satisfactory. The following is a description of a method of estimating guanine, based upon this reaction, which has been found to yield reliable quantitative results. The full description of the experiments which led up to the employment

[1] One important difficulty arises from the tendency for the formation of hypochlorous acid in mixtures of hydrochloric acid and potassium chlorate at temperatures only slightly below 100° C. Hypochlorites were found to accompany the guanidine in the subsequent stages of the analysis and to discharge the colour which develops when Marston's reagent is mixed with solutions containing guanidine.

of this method and its application to the estimation of the nucleo-cytoplasmic ratio in various organs will form the subject of a subsequent communication.[2]

METHODS.

1. *Reagents Required.*

2N Acetic Acid. This may be made up with sufficient accuracy by diluting 115 cc. of glacial acetic acid to one litre.

Washed Sand. Clean sea sand is digested on a boiling water-bath under a reflux condenser with strong hydrochloric acid for twenty-four hours. Then wash on a Buchner funnel with distilled water until the washings are free from chlorides, and dry the sand in an oven.

Normal Hydrochloric Acid.

Kieselguhr which has been washed with acids.

10% Sodium Hydroxide Solution.

One Molecular Disodium citrate Solution. This solution should yield a very faint pink when phenolphthalein is added to it and the mixture diluted by the addition of nine volumes of water. It may be prepared with sufficient accuracy by dissolving 36 grammes of trisodium citrate in 100 cc. of water.

30% Sodium Bisulphite Solution.

10% Cupric Sulphate Solution.

Half Normal Potassium Permanganate. This is prepared by dissolving 15·805 grammes of potassium permanganate crystals in 1 litre of freshly-boiled distilled water.

Alkaline Alcohol. This is prepared by diluting 10 cc. of a 14% solution of KOH to 100 cc. with absolute alcohol. The fine sediment of carbonate (which should only be present in traces) is allowed to settle overnight and the clear liquid decanted. This solution should be prepared the day before it is required, as it slowly assumes a yellow colour, which passes through the subsequent stages of the analysis and interferes with the final colorimetric estimation. If the mixture assumes a distinct yellow colour within twenty-four hours the alcohol should be redistilled over KOH.

Ten volumes per cent. Hydrogen Peroxide.

One-tenth Normal Hydrochloric Acid.

10% Potassium Ferricyanide Solution.

10% Sodium Nitroprusside Solution. This should be freshly made up every two months and the solution kept in a dark place.

18·63% Potassium Chloride Solution.

Standard Guanidine Solution. Prepared by dissolving 180 milligrammes of pure guanidine carbonate $(CN_3H_5)_2CO_3H_2$ in 200 cc. of tenth normal

[2] To be published in the Bulletin Series of the Commonwealth Council for Scientific and Industrial Research, Melbourne, Victoria, Australia.

hydrochloric acid. This contains one ten-thousandth of a gramme molecule of guanidine in 10 cc., or 4·2 milligrammes of guanidine nitrogen. This solution is preservatized by the addition of a few drops of toluene. It does not deteriorate.

2. *Preparation of the Tissues for Hydrolysis.*

Not less than 20 grammes of tissue is weighed, and then chopped into small pieces in a glass mortar with the aid of a pair of sharp scissors. Its own weight of washed sand is then added to the tissue, and the mixture ground into a pulp. The pulp is then quantitatively transferred, little by little to a large beaker containing a volume of boiling acidified alcohol corresponding to ten times the weight of tissue employed. Thus, if 20 grammes of tissue are employed the volume of acidified alcohol will be 200 cc. The acidified alcohol is prepared by adding 10 cc. of twice normal acetic acid to a litre of absolute alcohol. A thick-walled pyrex beaker should be employed, since constant stirring is essential to prevent bumping of the boiling mixture, and impacts of the stirring rod might otherwise break the beaker. After the transfer of the pulp to the beaker has been accomplished as completely as possible with the aid of a spatula, the mortar is washed out with a few cc. of acidified alcohol, the washings being transferred to the beaker. The mixture in the beaker is heated to brisk boiling for a few minutes, and when ebullition has ceased the fluid is passed through a hardened filter paper, taking care to leave as much of the residue in the beaker as possible.

The filtrate is rejected, and the residue is once more suspended in a volume of acidified alcohol corresponding to ten times the weight of the tissue. The contents of the beaker are then again heated to boiling, and after a few minutes' brisk boiling (with constant stirring) the fluid is passed through the same filter as before, as much of the residue as possible being, as before, retained in the beaker.

The contents of the beaker are now dried in a steam-heated oven, and then again pulverized in a mortar, the powdered mass being returned quantitatively to the beaker.[1] The residue is now extracted with a volume of fiftieth normal acetic acid, corresponding to ten times the original weight of tissue. The fiftieth normal acetic acid is made up by diluting 10 cc. of 2N acetic acid to 1 litre with distilled water. It is heated to boiling in a separate beaker, and then poured over the residue in the original beaker, which is heated, while stirring vigorously, until the mixture has boiled briskly for at least one minute. After the residue has settled the supernatant fluid is poured through the filter paper which was employed for the filtration of the alcoholic extracts, care being

[1] At this stage the residue should not be pulverized too finely or there will be difficulty in preventing it from collecting upon the filter paper, which diminishes the efficiency of the extraction (see below) and delays filtration. A Buchner filter may be employed for the aqueous and subsequent extractions, but in that case the residue on the original filter should be quantitatively returned, when dry, to the beaker containing the bulk of the residue.

taken, as before, to leave as much of the residue as possible in the beaker. The extraction with fiftieth normal acetic acid is repeated five times. After the fifth extraction with fiftieth normal acetic acid the residue is again extracted with the same volume of acidified alcohol, which is first heated to boiling in another beaker, then emptied into the beaker containing the residue, which, as before, is heated until brisk ebullition has occurred for at least one minute, the extract being filtered, after ebullition has ceased and the residue has subsided in the beaker, through the filter paper employed in the filtration of the previous extracts. The second extraction with acidified alcohol is repeated three times, or until the filtrate no longer yields a white cloud on mixing with an equal volume of water.

The residue is now extracted five times with ether, employing each time ten times the volume of ether which would correspond to the original weight of tissue. Finally, the residue is scraped off the filter paper back into the beaker, and the beaker, drained nearly dry, is placed in a steam-heated oven, or in an oven which has been heated to 100°C., and the gas turned off. When dry (it is not necessary to dry to constant weight) the whole of the residue is quantitatively transferred to a large weighing bottle, which may then be sealed with paraffin and kept until a convenient opportunity presents itself for completing the analysis.

The quantitative transferance of the contents of the beaker to the weighing bottle may be aided by a spatula and a long-handled camel's hair brush, taking care that no loose hairs from the brush become incorporated in the residue. The detachment of the last fine dust from the sides of the beaker may present difficulties, owing to the tendency of the dry particles to become electrified by the friction of the brush, and in consequence to fly apart from one another and resist collection by the brush into a small heap which can be transferred to the bottle. This difficulty may be overcome by washing down the sides of the beaker with a few cc. of ether, gently shaking the contents, with the beaker tipped to one side, until the powder is collected together in a small, compact mass, then pouring off the excess of ether. In a few moments the compacted residue is dry, and by inclining the beaker, and tapping its walls, may readily be tipped into the weighing bottle. The same device may be employed in the subsequent quantitative transfer of the contents of the weighing bottle to the Kjeldahl digestion flask, in which the hydrolysis is carried out.

3. *The Hydrolysis.*

The dried and extracted tissue and sand are quantitatively transferred to a 500 cc. pyrex Kjeldahl flask, and covered with a volume of normal hydrochloric acid, corresponding to ten times the weight of tissue. The flask is plunged into an oil-bath, which, as employed in these experiments, is a cylindrical can 7 inches high by 5 inches in diameter, on the open top of which are placed the copper rings taken from a water-bath. The can is supported on a tripod, and

heated by means of a micro-burner, the height of the flame being regulated by a screw clamp on the rubber tubing. One of the outer rings is perforated to admit a thermometer reading to 200°C., which is supported so that the bulb is about half-way down the can. The oil is an almost colourless paraffin. The neck of the Kjeldahl flask is inserted through the opening in the central ring, and supported at such a height that the level of the surface of the oil is well above that of the liquid inside the flask. A Hopkins condenser, made from a large pyrex test tube, is inserted in the neck of the Kjeldahl flask, the condenser tube and the neck of the flask being of the same length (8 inches). The flame is turned on full, and the time is noted at which the temperature of the oil-bath reaches 100°C. This is considered to be the beginning of hydrolysis, which is continued for eight hours, the flame being adjusted to maintain the temperature of the oil-bath at about 120°C.

The hydrolysate is filtered, while hot, through a 9 cm. S. and S. 597 filter paper, leaving the greater part of the sand in the Kjeldahl flask. The sand is then repeatedly washed with small lots of hot normal hydrochloric acid until the combined filtrate and washings nearly attain some convenient volume which is about double the original volume of normal hydrochloric acid which was employed in the hydrolysis. The filtrate is then made up to precisely the desired volume by the addition of normal hydrochloric acid solution. The filtrate constitutes the hydrolysable fraction, and contains all the guanine. The residue on the paper constitutes the unhydrolysable fraction, and the sand should be white and free from nitrogen.

4. *Removal of Humin Substances from the Hydrolysate.*

A portion of the hydrolysate (a few cc. is sufficient) is reserved for the estimation of its content of nitrogen. To the remainder is added Kieselguhr in the proportion of 4 grammes of Kieselguhr for every 100 cc. of hydrolysate. The Kieselguhr absorbs humin substances (10), which, if not removed, render the end-point in the subsequent titration with permanganate exceedingly difficult to determine. Furthermore, they are carried, in part, through the subsequent stages of the analysis, and communicate colour to the final solution, which is to be employed for the colorimetric estimation of guanidine.

After shaking the mixture of hydrolysate and Kieselguhr several times, it is allowed to stand for a few minutes, and then filtered through a rapid-filtering paper. The filtrate should be pale yellow, the colour of the original hydrolysate being dark brown.

5. *Precipitation of Purines from the Hydrolysate.*

A suitable volume (in animal tissues that corresponding to from 15 to 20 grammes of tissue, i.e., 300 to 400 cc.) is exactly neutralized by the addition of 10% NaOH solution, the amount required being ascertained by the preliminary titration of an aliquot (20 cc.). To the neutralized mixture is added

one-tenth of its volume of molecular trisodium citrate solution to buffer the reaction at approximate neutrality to phenolphthalein ($P_H = 8{\cdot}3$). To this, in turn, is added one-tenth of its volume of 30% sodium bisulphite solution, and the mixture is heated to boiling. While boiling a volume of 10% cupric solution, equal to that of the bisulphite solution, is slowly poured into the mixture, and boiling continued for two minutes after the last addition of cupric sulphate. The mixture is then allowed to cool, and centrifuged in a 100 cc. centrifuge tube until all of the precipitate has been collected at the bottom of the tube and the clear liquid decanted. The precipitate is then suspended three times in about 100 cc. of distilled water and recentrifuged.

After the first centrifugalization the precipitate may not be so closely packed that it can be drained perfectly dry without loss. Two or three cc. of fluid may have to be left in the tube on this account. By the second washing, however, the precipitate will pack firmly, and can then be drained dry without loss.

In each washing the first 10 cc. of water should be introduced in a jet from a wash-bottle, so as to break up the impacted precipitate, and the suspension of the precipitate in the wash-water is further aided by shaking before the centrifuge tube is filled to the top with water.

6. *Oxidation of the Purine Precipitate.*

Ten cc. of normal hydrochloric acid is delivered into the centrifuge tube, and the impacted precipitate broken up as much as possible by shaking. The tube is marked at the 10 cc. level, and then plunged into the oil-bath, heated to 120°C., and boiled for about five minutes, or until the precipitate, with the exception of a very small, finely divided residue, is dissolved. The tube is then withdrawn from the bath, made up to the original volume with distilled water, and again immersed in the bath until the contents are boiling. The tube is now withdrawn, and half normal potassium permanganate solution run in drop by drop, at the rate of about two drops per second, until a pronounced deepening of colour occurs and the mixture becomes reddish-orange. At this stage the oxidation of the guanine is complete, but if there is any doubt additional permanganate may be added drop by drop, more slowly, shaking between each addition, and waiting until any precipitate formed is redissolved before adding another drop. The appearance of a muddy precipitate which does not redissolve in thirty seconds constitutes a clearly discernible end-point. The volume of permanganate solution required is noted. The mixture is now poured into a porcelain casserole dish, together with washings from the tube, and evaporated to dryness on a water-bath.

7. *Estimation of the Guanidine Yielded by the Oxidation of the Purines.*

The residue derived from the oxidation of the tissue purines is extracted with 10 cc. of a mixture of one volume of 14% KOH with nine volumes of

absolute alcohol. The residue is broken up and mixed with the alkaline alcohol by means of a glass rod. Two cc. of ten volumes per cent. hydrogen peroxide are then slowly added, with constant stirring, to convert manganous compounds into manganese dioxide and oxidize alloxantin and related substances. The mixture should be tested at this point with moistened litmus paper, and if it is not alkaline an additional 10 cc. of alkaline alcohol and 1 cc. of hydrogen peroxide should be added. When effervescence has ceased 20 cc. of absolute alcohol are added, and the mixture stirred and then allowed to stand for not less than half an hour. The mixture is then filtered under suction through a small pad of asbestos in a Gooch crucible. The residue in the casserole is washed with 20 cc. lots of absolute alcohol until the combined filtrate and washings measure about 90 cc. The filtrate is then transferred to a 150 cc. Florence flask, washing out the filter flask with small lots of alcohol, and adding them to the contents of the Florence flask. Then acidify with 5 cc. of normal hydrochloric acid, and evaporate the contents of the flask to dryness in a steam-jacketted oven provided with sufficient outlet for the vapours of the alcohol.

The residue, containing the tissue-purine oxidation products, is taken up in 10 cc. of tenth normal hydrochloric acid with the aid of gentle heating. A slight insoluble residue remains suspended in the liquid, to which, after cooling, is added 1 cc. of 10% NaOH. If more than a trace of a precipitate of manganese dioxide appears at this point the estimation is a failure, and must be rejected, since guanidine is absorbed from alkaline aqueous solutions by freshly precipitated manganese dioxide, and as this precipitate must be removed before the colorimetic estimation can be performed, a proportion of the guanidine is removed with it. The probable causes of such a misadventure are the following:

(1) The employment of insufficient hydrogen peroxide to oxidize the manganous salts in the residue obtained after oxidation.
(2) Insufficient length of standing of the residue under alkaline alcohol before filtration.
(3) Insufficient stirring and breaking up of this residue in the alkaline alcohol.

The solution of oxidation products, thus rendered alkaline, is filtered through a sintered glass micro-filter (capacity 1·5 cc.) directly into a 25 cc. volumetric flask with the aid of suction applied by means of the arrangement depicted in the accompanying figure (figure 1). The flask is washed into the filter with successive small lots of water, until the volume of the combined filtrate and washings is somewhat less than 20 cc.

The standard is made up by introducing 10 cc. of standard guanidine solution into a 25 cc. volumetric flask. One cc. of 18·63% potassium chloride solution and 1 cc. of 10% sodium hydroxide solution are then added and a few cc. of water, until the volume of the contents of the flask is somewhat less than 20 cc., as in the case of the unknown.

While the solution and filtration of the final residue has been proceeding Weber's modification of Marston's reagent has been made up, and should be ready for use at the conclusion of these operations. The reagent is prepared by mixing 5 cc. each of 10% sodium nitroprusside solution, 10% potassium ferricyanide solution, and 10% sodium hydroxide in a graduated cylinder, and diluting the mixture to 60 cc. The mixture, at first very dark, rapidly fades to a light yellow. It is ready for use in half an hour.

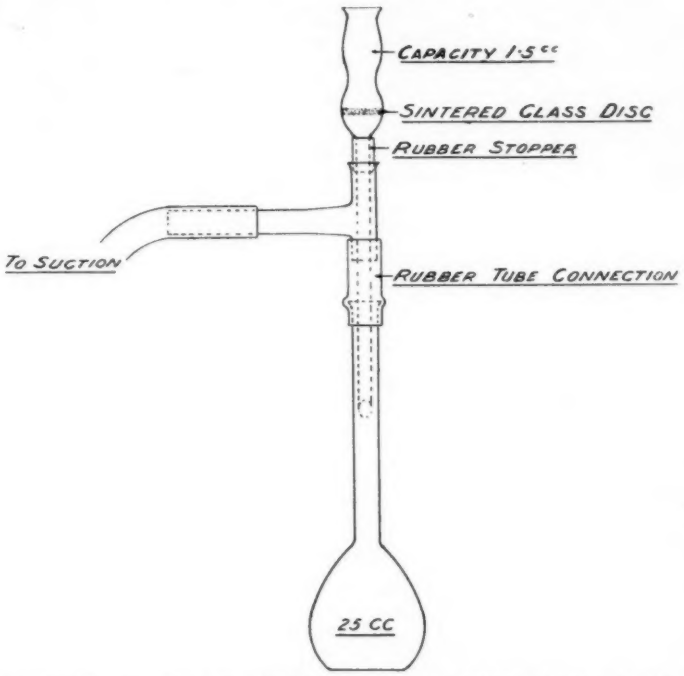


Figure 1. Showing apparatus for the filtration of the alcoholic solution of the oxidation products from purines.

To each of the volumetric flasks is added 5 cc. of this reagent, and the contents are mixed by shaking. The flasks are allowed to stand for half an hour, then diluted to the mark with distilled water, mixed, and compared in a Klett-Kober colorimeter reading 1/10 mm., the standard being set at 20 mm.

8. *Correction of Reading due to Difference of Heights of Columns.*

If the unknown and standard read the same there is obviously no correction. The concentration of the guanidine in the two solutions must be the same. If,

however, the columns are unequal in height then an error is introduced, arising from the fact that the reagent itself is not colourless. This might be avoided by employing one of the colorimeters in which this source of error is eliminated by employing a double column of reagent and unknown, on the one side, reagent and standard on the other, the unknown and reagent constituting the variable columns on the two respective sides. Unfortunately the colorimeter of this type which was available to us did not permit determination of the height of columns to within 1/10 mm., which is a degree of accuracy readily attainable in this estimation. It was found, however (by comparing the colours yielded by guanidine solutions of known and differing concentrations) that 1 mm. difference between the heights of the standard and unknown columns introduces an error equal to 0·34% of the standard (= 0·07 mg. of guanine hydrochloride, or 0·014 mg. of guanidine nitrogen). Thus, if, with the standard set at 20, the unknown reads 29·7 mm., then the uncorrected concentration of guanidine in the unknown would be 67·3% of that in the standard, but, owing to the greater height of the column on the unknown side, the concentration of guanidine in the unknown would be overestimated to the extent of $9{\cdot}7 \times 0{\cdot}34 = 3{\cdot}3\%$ of the standard, so that the correct concentration of guanidine in the unknown would be $67{\cdot}3 - 3{\cdot}3 = 64{\cdot}0\%$ of the standard. Similarly, if the column on the unknown side were 9·7 mm. shorter than the column of standard, the concentration of guanidine in the unknown would be underestimated, and to a like extent.

9. *Determination of the Total (Protein + Nucleic Acid) Nitrogen.*

The nitrogen in 1 cc. of the hydrolysate is determined by the micro-Kjeldahl method of Folin.

The original filter paper and contained unhydrolyzed fraction are transferred to a Kjeldahl flask and digested with a suitable volume of a mixture of equal parts of Folin's oxidizing-hydrolyzing mixture (4) and sulphuric acid. For the residue obtained from 25 grammes of animal tissue 40 cc. are sufficient. It is desirable to use the least amount of this mixture, because the success of the subsequent Nesslerization may be interfered with by the high concentration of salts. For this reason also the filter paper employed in the filtration of the original hydrolysate should not exceed 9 cm. in diameter. When the fluid becomes colourless, and has been sufficiently cooled, it is diluted and then filtered free from precipitated silicates. The filter paper is washed, the washings being collected in the same vessel, until the combined volume of filtrate and washings is nearly 250 cc. The filtrate and washings are then diluted to exactly that volume and thoroughly mixed. From this solution 5 cc. is measured out into a small beaker, diluted, and titrated with 10% sodium hydroxide to neutrality to phenolphthalein. A fresh 5 cc. sample is introduced into another small beaker, neutralized with the ascertained proportion of 10% sodium

hydroxide, and 5 cc. of 10% sodium hydroxide is added, in addition to that required for neutralization. The mixture is filtered through a small soft paper into a 200 cc. volumetric flask, and the paper is repeatedly washed into the same flask. The contents of the flask are diluted to about 150 cc. with water, 20 cc. of Nessler's reagent added in the usual manner, and the mixture diluted to exactly 200 cc. The standard is prepared by introducing 1 cc. of a standard ammonium sulphate solution containing 1 mg. of nitrogen per cc. into a 200 cc. volumetric flask, diluting to about 150 cc., adding 5 cc. of 10% sodium hydroxide, and 20 cc. of Nessler's reagent, and diluting to mark. These solutions must be compared quickly, because, owing to the high concentration of salt in the unknown, it tends to become cloudy on standing. The total (coagulable protein + nucleic acid) nitrogen is the sum of the amounts of nitrogen found in the hydrolysate and in the unhydrolyzable fraction.

10. *Calculation of the Nucleo-cytoplasmic Ratio.*

From the yield of guanidine nitrogen, multiplying by 5, is obtained the nucleic-acid nitrogen. The ratio:

$$\frac{\text{Nucleic Acid Nitrogen}}{\text{Total Nitrogen} - \text{Nucleic Acid Nitrogen}}$$

which is equivalent to

$$\frac{\text{Nucleic Acid Nitrogen}}{\text{Coagulable Protein Nitrogen}}$$

may be regarded as a measure of the relative magnitudes of the ratios of nuclear to cytoplasmic living material in an animal or plant.

EXPLANATION OF CERTAIN STEPS IN THE METHOD.

1. *Preparation of the Tissues for Hydrolysis.*

The method adopted for this purpose will depend upon our conception of the proper measure of living cytoplasm. If we grant that nucleic acid affords a reasonable relative measure of the mass of nuclear material in tissues, what should we employ as a relative measure of cytoplasm? LeBreton and Schaeffer employ total nitrogen, but this will include many metabolites and substances such as creatine, phospholipines, etc., which perform special functions in certain tissues not necessarily related in any way to their cytoplasmic mass. The constant characteristic of cytoplasm is the presence of protein, and the author would suggest that coagulable protein nitrogen constitutes a relative measure of cytoplasmic mass, which is theoretically preferable to total nitrogen. It is admitted that the percentage of water, and therefore of protein, in different tissues may vary greatly, but it has yet to be shown that the *volume* of cells of unlike function is of such importance in relation to nuclear mass, as the *mass*

of the colloidal complex, holding water in its interstices, which constitutes the active portion of the cytoplasm. In other words, we have in cytoplasm the same variation of the relative proportions of different constituents as that to which Conklin drew attention in the nucleus (3). In this dilemma we have only one resource, namely, to settle upon some one highly characteristic constituent of each structure, and employ the ratio of these constituents as a relative measure of the ratio of the physiological (as contrasted with the visible or anatomical) structures. The use of coagulable protein as a measure of cytoplasm has the further advantage that protein is probably absent from the nucleus (1).

It is recognized that certain proteins in tissues are not coagulable by heat. By far the greatest proportion of protein of this type in the animal body, however, is furnished by the collagen of fibrous connective tissue, which is of structural (sclerous) rather than cellular significance, and may no longer display the typical dependence of cells upon their individual nuclei. To sum up: Whatever uncertainties this measure may involve, they are, at all events, less than the uncertainties involved in other measures of cytoplasmic mass, and pending fuller knowledge it would appear the most practicable standard to employ.

The use of boiling fiftieth normal acetic acid solution as the coagulating agent is based upon the observation that acetic acid of this order of concentration brings about complete coagulation of blood serum proteins at boiling temperature without inducing appreciable hydrolysis (12). The repeated extractions with fiftieth normal acetic acid remove the non-protein nitrogenous extractives, such as creatin. The alcoholic and ether extractions remove nitrogen-containing fats (phospholipins). Practically the whole of the residual nitrogenous substances must consist of proteins and nucleic acids, together with small amounts of oxypurines, etc., which cannot be sufficiently large to interfere appreciably with the accuracy of the estimate of coagulable protein in terms of nitrogen.

The tissue is first extracted twice with acidified alcohol to permit the subsequent filtration of the fiftieth normal aqueous acetic acid extracts. If a considerable proportion of the fat is not previously removed in this way the aqueous extracts are impossible to filter, owing to the presence of emulsified fat, which clogs the pores of the filter-paper. Nor can they be centrifuged without loss of protein nitrogen, because the emulsion of fat holds a variable proportion of insoluble nitrogenous material in suspension. The procedure recommended yields rapid-filtering extracts at every stage. The reduplicability of results, employing this method of extractions, is shown by the following experiment:

Sheep's liver was minced, passing it four times through the mincing machine to ensure thorough mixing. Four 15 gramme samples were weighed out and extracted in the manner described above. The following were the results obtained:

Sample. No.	Weight of dry residue after subtraction of weight of 15 grammes of sand similarly extracted.	Nitrogen content of residue.
1	2·47 grammes	388 milligrammes
2	2·47 „	386 „
3	2·49 „	384 „
4	2·53 „	403[1] „

2. *The Hydrolysis.*

In the hydrolysis we have relied upon the careful estimations of LeBreton and Schaeffer of the yield of amino-purines from tissues hydrolyzed wth various strengths of sulphuric acid. They obtain the maximum yield with 3% sulphuric acid, corresponding to two-thirds normal. We have not hesitated to employ hydrochloric acid of somewhat greater equivalent concentration, because we have been concerned only with guanine, not with adenine, and repeated experiments designed to test this point have shown that guanine may even be evaporated to dryness in normal hydrochloric acid solution without appreciable loss.

3. *Precipitation of Purines from the Hydrolysate.*

Under the conditions attending our estimations we have found the P_H at which precipitation of purines by Kruger's method (6) is carried out to be of considerable importance. Neutralization with 10% sodium hydroxide, which is necessary to economize volume, leaves room for considerable variation in the final P_H. The character of the precipitate obtained is correspondingly variable, for at the higher alkalinities much cuprous oxide is precipitated, which constitutes an embarrassment in the subsequent washing of the precipitate (owing to its tendency to form colloidal suspensions), and, in so far as it passes through the washings to the precipitate which is subjected to oxidation, it gives rise to further difficulties, necessitating the employment of large excesses of permanganate, masking the end-point by the formation of precipitates, and rendering it difficult to discern the end-point.

Since it was found that quantitative precipitation of the purines occurred at neutrality to phenolphthalein ($P_H = 8·3$), it was sought to buffer the reaction of the purine solution at this P_H before undertaking the precipitation. Trisodium citrate was chosen as a suitable salt to accomplish this purpose, but its use was attended with several unanticipated advantages. This salt, as in Benedict's qualitative reagent for sugar (2), retains cupric hydroxide in solution, but it also, under the circumstances of this analysis, prevents the precipitation of cuprous oxide. At the moment of precipitation this is of great advantage, because the excessive foaming, which is caused by the formation of colloidal or finely divided cuprous oxide in the mixture, is prevented, and the

[1] This was the only sample in which any considerable proportion of the residue was transferred to the filter. The effect of this is evidently to diminish the efficiency of the extractions leading to over-estimation of the protein nitrogen.

copper sulphate may be added quite quickly without withdrawal of the flame or incurring any danger of boiling over. The subsequent difficulties attending the oxidation of the purines in the presence of excess of cuprous oxide are, of course, avoided. The yield of guanine is quantitative.

4. *Oxidation of the Purine Precipitate.*

The end-point of the reaction is indicated in pure solutions of guanine in normal hydrochloric acid by the appearance of a pink colour which does not fade for 30 seconds after the addition of the last drop of half normal potassium permanganate. The addition of two or three drops in excess of this results in the formation of a muddy precipitate which does not redissolve on standing. The addition of this excess of permanganate does not appreciably diminish the yield of guanidine, although large excesses do so. The former end-point (colour) is somewhat indistinct in the oxidation of the copper precipitate of purines, on account of the presence of adenine (see below). In event of doubt the latter end-point (appearance of a muddy precipitate) may be employed as an alternative.

The specificity of the reaction was established by treatment of various purines and related substances with permanganate in hydrochloric acid solution and testing the products with Marston's reagent. The following substances failed to furnish any substances yielding colour with Marston's reagent: adenine, xanthine[3], hypoxanthine, inosine[4], uric acid. Arginine yields a colour which approximates to that which would be expected if the yield of guanidine from this substance were quantitative, but the colour fades (50% in an hour). This reaction was discovered by Mr. Hedley R. Marston during his investigation of the reactions of his reagent with various derivatives of guanine, but has not previously been published. Mr. Marston is at present engaged in further examination of this reaction, with a view to its employment in the quantitative estimation of arginine in protein hydrolysates.

5. *The Effect of Oxidation Products of Purines upon the Colour Value of Guanidine treated with Marston's Reagent or Weber's Modification of Marston's Reagent.*

Certain substances interfere in greater or lesser degree with the development of colour when Marston's reagent is mixed with guanidine in alkaline solutions. The effect of hypochlorites has already been alluded to. Sulphides

[3] A sample of xanthine obtained from a commercial firm yielded a slight coloration. After purification by solution in a minimal amount of 1% ammonia and reprecipitation with excess of hydrochloric acid the substance no longer furnished any products yielding colour with Marston's reagent.

[4] A pure sample, very kindly furnished to me some years ago by Dr. P. A. Levene.

similarly, even in traces, discharge the colour from the mixtures.[5] Among organic substances if creatinine is present the colour forms and then rapidly fades, a phenomenon which is probably attributable to the fact that creatinine is a reducing agent, and competes with guanidine for the available oxygen in Marston's reagent, precisely as it competes with sugar in the reduction of Fehling's solution (7). Since creatine is converted into creatinine on evaporation in acid solutions, the presence of creatine in the solution of purine oxidation products would render the final estimation impossible, although creatine itself does not influence the development of colour with Marston's reagent. The preliminary extraction of the tissues with fiftieth normal acetic acid, of course, removes creatine and creatinine from the residues, even if the subsequent isolation of the purines by copper precipitation did not also obviate this source of error. It is necessary to draw attention to this effect of creatinine, however, since it appears to have been overlooked previously, and other estimations involving the determination of guanidine by Marston's method might be affected by its presence.

Certain products which arise from the oxidation of purines by hydrochloric acid and potassium permanganate considerably diminish the final colour value obtained when alkaline guanidine solutions are mixed with Marston's reagent or Weber's modification of this reagent. In the case of Marston's reagent, which is made up with potassium ferrocyanide and hydrogen peroxide, the magnitude of the effect is variable, depending considerably upon the length of time the reagent has stood after the addition of hydrogen peroxide. This apparently arises from the fact that the hydrogen peroxide is continually decomposing from the moment of mixture of the constituents of the reagent, and the oxidation potential of the system is therefore falling. Colour development is delayed by the purine oxidation products, and becomes stabilized at levels which vary with the oxidation potential of the mixture at the moment. This is shown by the fact that if Marston's reagent is added to the guanine solution immediately after mixing the depression of colour value due to the presence of alloxan is at a minimum, and increases with the period of standing of the mixture. The following are illustrative determinations, the readings being taken in each case half an hour after the addition of the reagent to the solutions. The readings are expressed in percentages of the standard guanidine solution (without alloxan) to which Marston's reagent had been added one hour after mixing its constituents. To five of the standard guanidine solutions were added 20 mg.

[5] This renders it impossible to free the copper-purine precipitate from copper by H_2S, as the filtrate cannot be rendered sufficiently free from sulphur to enable the successful employment of Marston's reagent to estimate the guanidine. While the precipitates were contaminated with excess of cuprous oxide this difficulty was serious, but the employment of citrate in the solution from which the purines are precipitated rendered the separation of copper from the precipitate unnecessary.

èach of alloxan, prepared by the oxidation of uric acid.[6] The other five received no addition.

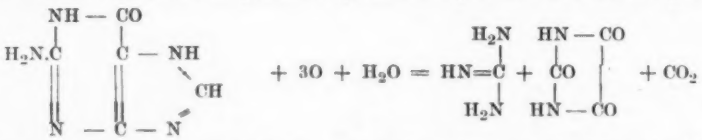
TABLE 1.

Time after mixing the constituents of Marston's reagent.	Per cent. of one hour standard. With alloxan.	Without alloxan.	Difference due to alloxan in per cent. of standard made up at same time.
5 minutes	157·5	196·1	19·7
15 "	105·8	130·7	19·1
25 "	94·6	118·2	20·0
35 "	75·7	100·0	24·3
65 "	71·2	100·0	28·8

The alteration of the colour-producing capacity of Marston's reagent with guanidine is so rapid during the first minutes after mixing (during which energetic effervescence of oxygen is occurring) that the reagent cannot be considered stabilized in any degree calculated to yield comparable results from one set of determinations to another until 45 minutes have elapsed after mixing. By this time the depression of colour value, due to the presence of 20 mg. of alloxan, is 24·3% of the colour yielded by a standard containing 5·9 mg. of guanidine (= 4·2 mg. of guanidine nitrogen) in a final volume of 25 cc.

When this experiment is repeated with Weber's modification of Marston's reagent (potassium ferricyanide instead of potassium ferrocyanide and hydrogen peroxide) the colour development is complete in the pure guanidine standard within two or three minutes, but that containing twice the molecular equivalent of alloxan has only just perceptibly begun to redden at one minute, and for at least five minutes the colour of the solution is changing. At thirty minutes, however, the colour has reached a constant value somewhat below that of the standard (figure 2).

A product from uric acid which may give rise to this effect is indicated by these experiments. The oxidation products from guanine, however, produce the same effect upon colour development. From the observations of Strecker it is believed that the oxidation of guanine by chlorine pursues the following course:



with the production of guanidine, parabanic acid, and carbon dioxide. It would appear probable, therefore, that the depression of colour value in solutions of the oxidation products of guanine might be due to parabanic acid. Two preparations of parabanic acid were tested, one prepared synthetically from oxalyl urea, the other by oxidation of uric acid with boiling nitric acid; both

[6] The alloxan employed in these experiments was not especially purified and contained traces of alloxantin, to which, as we shall see, the colour-depressing effect was probably due.

failed, however, to yield any effect upon colour development in guanidine solutions to which Marston's reagent or Weber's modification were added. The nature of the product which leads to the depression of colour value in solutions of guanidine derived from the oxidation of guanine, therefore, remains for the present undetermined.

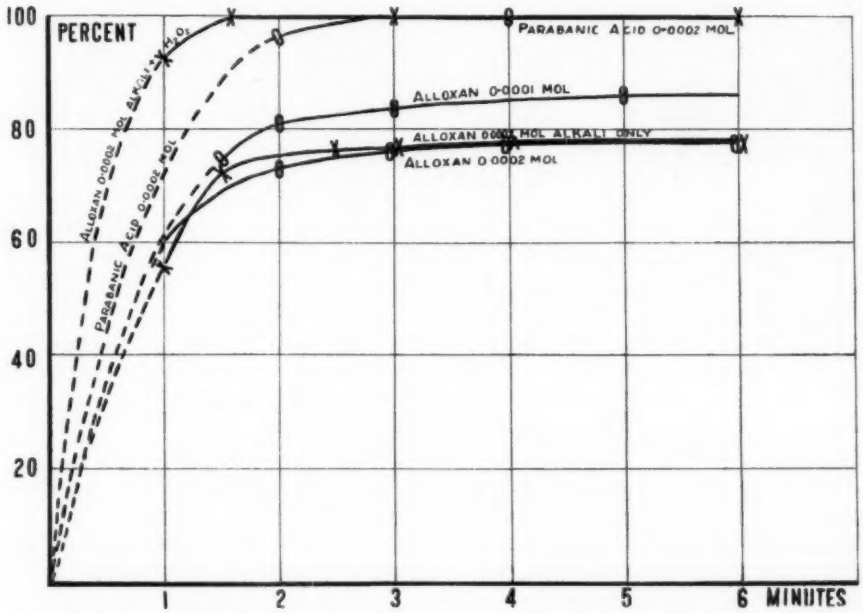


Figure 2. Showing comparative rates of colour development in the presence of 0·0001 and 0·0002 mol. of alloxan, 0·0002 mol. of alloxan treated with alkaline alcohol only for half an hour and then acidified and evaporated to dryness; 0·0002 mol. of alloxan similarly treated with alkaline alcohol and 2 cc. of 10 vols. H_2O_2, and 0·0002 mol. of parabanic acid. The marks X and O indicate the actual determinations, all in terms of percentage of a standard guanidine mixed with Weber's modification of Marston's reagent one half-hour previously.

The substances derived from the oxidation of guanine and of uric acid which depress the colour value and delay the development of colour in these mixtures are, however, deprived of this property by oxidation with alkaline hydrogen peroxide. This is shown in the accompanying figure (figure 2), in which the rate of colour development in guanidine standards containing two ten-thousandth molecular equivalents of impure alloxan (containing alloxantin) with and without preliminary treatment with alkaline peroxide are compared. The rate, after oxidation with peroxide, is indistinguishable from the rate of colour development without alloxan, and the final depth of colour attained is the

same. Since alloxantin would very readily be oxidized by this procedure, it is probable that the effects of alloxan containing alloxantin are entirely attributable to the latter substance. Some similar intermediate product of oxidation is undoubtedly responsible for the depression of colour values brought about by oxidation products of guanine. The treatment of the alkaline alcoholic extract of the oxidation products with hydrogen peroxide, therefore, accomplishes the two-fold purpose of converting manganous compounds which are slightly soluble in alcohol into manganese dioxide, which is insoluble, and of oxidizing inhibiting substances which would otherwise depress the colour values in the final colorimetric estimations.

6. *The Volume of Half Normal Potassium Permanganate Solution required to Oxidize the Purine Precipitate.*

The volumes of half normal potassium permanganate solution consumed by various purines and related substances when titrated under the conditions specified above and to the end-point defined by a pink flush, which persists for thirty seconds, are given in the accompanying table. The "blank," that is, the volume of permanganate solution consumed by the solvent (10 cc. of normal HCl), is subtracted from each of the observed titration figures. This is 0·15 cc. for the longer titrations (guanine, uric acid), 0·20 cc. for the shorter titrations.[7]

TABLE 2.

Substance.	Volume of half-normal potassium permanganate consumed by one ten thousandth gram molecule of substance (equivalent to 20·55 mg. of guanine hydrochloride).
Guanine hydrochloric	1·50 cc.
Uric acid[8]	0·43 cc.
Xanthine[9]	0·35 cc.
Arginine	0·20 cc.
Adenine	0·00 cc.
Hypoxanthine	0·00 cc.
Alloxan	0·00 cc.
Parabanic acid (from uric acid) ..	0·05 cc.

The case of adenine is peculiar. A red flush appears in a solution of 20·2 milligrammes of adenine sulphate ($[C_5H_5N_5]_2H_3SO_4.2H_2O$) in 10 cc. of

[7] When little permanganate is used the whole titration is conducted practically at boiling temperature, and at this temperature 0·20 cc. of half normal permanganate are required to communicate lasting colour to 10 cc. of normal hydrochloric acid. At the lower temperatures prevailing at the end of longer titrations this falls to 0·15 cc.

[8] The uric acid employed was "Pfanstiehl" uric acid, prepared by The Special Chemicals Company, Highland Park, Ill., U.S.A. The titre given is calculated from the formula: titre in cc.$=25(x + x^2)$ where x is the weight of uric acid in grammes. It was ascertained from a large number of estimates that this formula applies for quantities of uric acid varying from 10 to 100 milligrammes dissolved in 10 cc. of normal hydrochloric acid which is initially heated to boiling.

[9] The estimate for xanthine is a minimal one, because under the specified conditions of the titration the whole of the xanthine is not dissolved when the end-point has been attained.

normal hydrochloric acid upon the addition of 0·2 cc. of half normal permanganate to the boiling solution. The red is, however, not the rose-red typical of excess of permanganate, but an orange-red which does not fade on prolonged standing. Further addition of permanganate leads to progressive deepening of the colour, which at about 1·0 cc. is a deep red with an orange tint. The precise point at which a muddy precipitate appears varies with the temperature, but if the solution is allowed to cool nearly to room temperature before the titration is concluded a maximum titre of 1·75 cc., corresponding to 1·60 cc. of permanganate actually neutralized by the adenine, is reached before the appearance of a muddy precipitate. This corresponds to eight equivalents of permanganate per gramme molecule of adenine. The solution thus obtained is unstable, and on heating the whole of the coloured matter is precipitated, and when filtered yields a cinnabar-red residue upon the paper and a colourless filtrate which contains no adenine. The precipitate is soluble in normal alkali, yielding a red solution. These facts point to its being a compound of adenine with an oxide or chloride of manganese, analogous to the compounds with silver and copper oxides or with the chlorides of mercury, gold, and platinum.

It is the presence of adenine in the purine-copper precipitate which renders the determination of the end-point of the oxidation with permanganate rather difficult. Instead of the sudden appearance of a red flush, as in solutions containing only guanine, we have a gradually deepening orange. The deepening of colour is relatively sudden, however, and with practice can be detected to within 0·1 cc. and reproduced in successive titrations. In a mixture of 20·55 mg. of guanine hydrochloride with 20·2 mg. of adenine sulphate in 10 cc. of normal hydrochloric acid, the deepening of colour occurs at 1·60 cc., that is, at 0·05 cc., prior to the completion of the oxidation of guanine (allowing for 0·15 cc. consumed by the solvent). The oxidation of guanine is therefore completed before the compound with adenine is formed, and the colour of this compound may be employed as an indicator of the completion of the oxidation. If, however, this end-point is not employed, if the solution be allowed to cool, and the successive drops of permanganate are added very slowly, a muddy precipitate occurs when the adenine has combined with permanganate corresponding to eight equivalents per gramme molecule of adenine. The earlier end-point is preferable because it employs less permanganate and involves less danger of the carrying over of manganese into the final solution employed for colorimetric comparison with the standard.

The precipitate of cuprous purine compounds from the tissue hydrolysate will consist of guanine and adenine in equimolecular proportions, together with any of the oxy-purines, hypoxanthine, and uric acid, which may not have been removed in the extractions to which the tissue is subjected before analysis. One ten-thousandth equivalent of potassium permanganate (in terms of available oxygen) is contained in 0·20 cc. of half normal solution. The volume of

permanganate consumed by guanine corresponds to 7·5 equivalents per gramme molecule of guanine. The cuprous oxide combined with a gramme molecule of guanine would consume two equivalents (corresponding to one atom of oxygen), and that combined with the adenine a like amount. If the earlier end-point (orange-red colouration) is employed, adenine consumes no permanganate. If the solution be allowed to cool in the final stages of the addition of permanganate, and the second end-point (muddy precipitate) is employed, adenine, as stated, consumes eight equivalents of permanganate per gramme molecule. If any oxy-purines are present a corresponding excess of permanganate over the quantities indicated by these figures would be consumed. In the case of uric acid the permanganate would be shared almost equally between the copper and the purine (0·40 cc., as compared with 0·43 cc. per ten-thousandth gramme molecule of uric acid), and wholly consumed by the copper in the case of hypoxanthine.

The oxy-purines originally present in the tissue must undoubtedly be reduced by the preliminary extractions to which the tissues are subjected before analysis. We cannot state that oxypurines are wholly removed from other tissues by these extractions, but in the case of liver tissue which has been removed some time from the body of the animal (in which uric acid is commonly present to a less extent than in other tissues [5]), the extractions appear to remove oxy-purines completely, as the following comparative estimates reveal:

Example 1.

In the hydrolysate from a sample of yeast nucleic acid the guanine found corresponded to 1·20 cc. of permanganate. The first end-point was employed.

The copper combined with this amount of guanine would have consumed 0·32 cc. of permanganate.

The copper combined with the corresponding amount of adenine would have consumed 0·32 cc. of permanganate.

The adenine, at the end-point employed, would have consumed no permanganate.

The solvent (10 cc. of normal hydrochloric acid) would have consumed 0·15 cc. of permanganate.

Total calculated requirement, 1·99 cc.

Half normal permanganate actually required, 1·90 cc.

Example 2.

In a portion of the hydrolysate derived from sheep's liver, the guanine found corresponded to 1·35 cc. of permanganate. The second end-point was employed.

The copper combined with this amount of guanine would have consumed 0·36 cc. of permanganate.

The copper combined with the corresponding amount of adenine would have consumed 0·36 cc. of permanganate.

The adenine, at the end-point employed, would have consumed 1·44 cc. of permanganate.

The solvent (10 cc. of normal hydrochloric acid) would have consumed 0·15cc. of permanganate.

Total calculated requirement, 3·66 cc.

Half normal permanganate actually required, 3·65 cc.

Were this result generally applicable it might appear that with the preliminary extractions employed in the estimation of the nucleo-cytoplasmic ratio, the nitrogen content of the copper purine precipitate might be employed, as in Le Breton and Schaeffer's estimations, as a measure of the amino-purine content of the tissues. But there is no advantage either of accuracy or of convenience in estimating nitrogen rather than guanine in this precipitate, and the applicability of the former method is limited to those tissues from which total removal of oxy-purines by the preliminary extractions has been demonstrated, and is wholly dependent for its accuracy upon the thoroughness of the extraction. The interpretation of the nitrogen-content of the amino-purine precipitate is always open to doubt, while the guanine content cannot be derived from any other source than pre-formed nucleic acid.

7. *The Phosphoric Acid Content of the Hydrolysate.*

Since the preliminary extractions with acidified alcohol, fiftieth normal acetic acid and ether would remove phospholipins and a large proportion, if not the whole, of the inorganic phosphates from the tissues, the question suggests itself whether the phosphoric acid content of the hydrolysate may not be used quite as conveniently as the guanine content, as a measure of the nucleic acid present in the tissues. This, however, overlooks the important factor introduced by proteins containing phosphoric acid, of the type of casein, from which the phosphoric acid is not removable except by hydrolytic cleavage of the protein. The phosphoric acid content of the hydrolysate is, in fact, considerably in excess of the quantity which would be yielded by the nucleic acid equivalent to the guanine actually found. The discrepancy may be illustrated by the following examples:

Example 1.

A sample of hydrolysate from liver contained 0·0000894 gramme molecules of guanine.

This corresponds to four times the amount, or 0·000358 gramme atoms of phosphorus derived from nucleic acid.

This equals 11·1 milligrammes of phosphorus.

Amount of phosphorus actually present[10] was 16·1 milligrammes.

[10] Estimated, after evaporation and ignition, by Brigg's method.

Example 2.

A sample of hydrolysate from liver contained 0·0000902 gramme molecules of guanine.

This corresponds to 0·000361 gramme atoms of phosphorus derived from nucleic acid.

This equals 11·2 milligrammes of phosphorus.

Amount of phosphorus actually present was 15·3 milligrammes.

Example 3.

A sample of hydrolysate from liver contained 0·0000949 gramme molecules of guanine.

This corresponds to 0·00038 gramme atoms of phosphorus derived from nucleic acid.

This equals 11·8 milligrammes of phosphorus.

Amount of phosphorus actually present was 16·1 milligrammes.

No attempt has been made to ascertain whether this excess of phosphorus is present in the extracted tissues in the form of phospho-proteins or of inorganic phosphates which are insoluble in dilute acetic acid, but the existence of the discrepancy illustrates the difficulty, to which allusion was made in the introductory paragraphs, of estimating nucleic acid in terms of the phosphoric acid which it liberates when hydrolyzed.

8. *The Addition of Potassium Chloride to the Guanidine Standard.*

It has been pointed out by Weber (17) that the colour values obtained in solutions of known guanidine content is diminished by the presence of inorganic salts. The salts present in the final solution of the unknown must consist almost wholly of potassium chloride derived from the neutralization of the potassium hydroxide contained in the alkaline alcohol by the hydrochloric acid, which is subsequently added to the alcoholic extract. The amount of KCl thus introduced is 0·1863 grammes. My own measurements have shown that the addition of this amount of KCl to standard guanidine solution containing 4·2 milligrammes of guanidine nitrogen in a final volume of 25 cc. reduces the colour yielded by Weber's modification of Marston's reagent by 4·6 per cent. It must therefore be added to the standard in order to render the colour value of the guanidine contained in the standard comparable with that of the guanidine which is contained in the unknown.

ILLUSTRATIVE EXAMPLES OF THE APPLICATION OF THE METHOD TO THE ESTIMATION OF NUCLEO-CYTOPLASMIC RATIOS AND THE RECOVERY OF GUANINE ADDED TO TISSUE HYDROLYSATES.

Experiment No. 1.

A male mouse weighing 25·5 grammes, and between 20 and 30 weeks of age, was killed with chloroform and subjected to extraction and hydrolysis as outlined

above.[11] The hydrolysate and washings were diluted to a volume of 500 cc., and then divided into three parts: A, volume 150 cc., no addition; B, volume 150 cc., 20·55 mg. of guanine hydrochloride added; C, volume 100 cc., 41·1 mg. of guanine hydrochloride added.

A	Guanidine nitrogen found in sample	1·361 mg.
	Corresponding to guanine hydrochloride[12]	6·662 „
	Corresponding to nucleic acid nitrogen	6·807 „
B	Guanidine nitrogen found in sample	5·531 „
	Corresponding to guanine hydrochloride	27·11 „
	Subtracting guanine present in A	20·45 „
	Added guanine recovered	99·5%
C	Guanidine nitrogen found in sample	9·195 mg.
	Corresponding to guanine hydrochloride	45·07 „
	Subtracting two-thirds of guanine present in A	40·63 „
	Added guanine recovered	98·8%
From A		
	The total nucleic acid nitrogen in the mouse was	22·69 mg.
	The total coagulable protein plus nucleic acid nitrogen was	496·6 „
	Subtracting nucleic acid nitrogen, the protein nitrogen was	473·9 „

$$\text{Nucleo-cytoplasmic ratio} = \frac{22{\cdot}69}{473{\cdot}9} = 0{\cdot}048.$$

This experiment, although it yielded consistent results and almost quantitative recovery of added guanine, was not carried out under ideal conditions, because the volume of hydrolysate employed to estimate the purines in the tissue, without added guanine, was too small. This led to a large correction of the reading, due to the difference in the height of the columns of the standard and the unknown. It is, of course, desirable that this correction should be kept as small as possible.

Experiment No. 2.

A male mouse, weighing 29·0 grammes, and 551 days old, was killed with chloroform and subjected to extraction and hydrolysis. The hydrolysate and washings were diluted to a volume of 500 cc., and divided into two parts: A, volume 300 cc., no addition; B, volume 100 cc., 20·55 mg. of guanine hydrochloride added.

A	Guanidine nitrogen found in sample	2·969 mg.
	Corresponding to guanine hydrochloride	14·53 „
	Corresponding to nucleic acid nitrogen	14·845 „
B	Guanidine nitrogen found in sample	5·145 „
	Corresponding to guanine hydrochloride	25·17 „
	Subtracting one-third of guanine present in A	20·33 „
	Added guanine recovered	98·9%

[11] For accurate estimations of the nucleo-cytoplasmic ratios in whole animals the animal should be deprived of food until the alimentary canal is virtually empty, so as to avoid inclusion of foodstuffs in the estimate. For the mouse this would be about 12 hours (13). In this and the next following experiment this precaution was omitted because they were designed rather to investigate the recovery of added guanine than the actual values of nucleo-cytoplasmic ratios. The values obtained must therefore be regarded as provisional.

[12] Calculated as $C_5H_5N_5O.HCl + H_2O$.

From A

The total nucleic acid nitrogen in the mouse was	24·722 mg.
The total coagulable protein plus nucleic acid nitrogen was	657·9 „
Subtracting nucleic acid nitrogen, the protein nitrogen was	633·2 „

$$\text{Nucleo-cytoplasmic ratio} = \frac{24.72}{633.2} = 0.039.$$

Experiment No. 3.

A sample of sheep's liver, weighing 30·0 grammes, was subjected to extraction and hydrolysis. As the liver was obtained from a butcher's shop the age of the sheep and time which had elapsed since it was killed are unknown.[13] The hydrolysate and washings were diluted to a volume of 500 cc., and divided into two parts: A, volume 350 cc., no addition; B, volume 100 cc., 20·55 mg. of guanine hydrochloride added.

A	Guanidine nitrogen found in sample	3·788 mg.
	Corresponding to guanine hydrochloride	18·54 „
	Corresponding to nucleic acid nitrogen	18·94 „
B	Guanidine nitrogen found in sample	5·330 „
	Corresponding to guanine hydrochloride	26·08 „
	Subtracting two-sevenths of guanine present in A	20·78 „
	Added guanine recovered	101·1%

From A

The total nucleic acid nitrogen in 30 grammes of liver was	27·057 mg.
The total coagulable protein plus nucleic acid nitrogen was	697·7 „
Subtracting nucleic acid nitrogen, the protein nitrogen was	670·6 „

$$\text{Nucleo-cytoplasmic ratio} = \frac{27·06}{670·6} = 0.040.$$

Experiment No. 4.

Another sample of sheep's liver, weighing 30 grammes, and similarly obtained, was subjected to extraction and hydrolysis. The hydrolysate and washings were diluted to a volume of 500 cc., and divided into two parts: A, volume 300 cc., no addition; B, volume 100 cc., 20·55 mg. of guanine hydrochloride added.

A	Guanidine nitrogen found in sample	3·755 mg.
	Corresponding to guanine hydrochloride	18·37 „
	Corresponding to nucleic acid nitrogen	18·78 „
B	Guanidine nitrogen found in sample	5·481 „
	Corresponding to guanine hydrochloride	26·82 „
	Subtracting one-third of guanine present in A	20·69 „
	Added guanine recovered	100·7%

From A

The total nucleic acid nitrogen in 30 grammes of liver was	31·292 mg.
The total coagulable protein plus nucleic acid nitrogen was	621·6 „
Subtracting nucleic acid nitrogen, the protein nitrogen was	590·3 „

$$\text{Nucleo-cytoplasmic ratio} = \frac{31·29}{590·3} = 0.053.$$

[13] To obtain correct values of the nucleo-cytoplasmic ratio for individual organs (as contrasted with whole animals) only tissues from which blood has been removed by perfusion with Ringer's solution should be employed. The numerical data given here for liver tissue, therefore, which were sought rather to illustrate the method than to obtain correct values of the ratio, are to be regarded as provisional and approximate.

The average proportion of added guanine which was recovered in these five estimations was therefore 99·8%, with a maximum deviation from the true estimate of 1·2%.

SUMMARY.

A method of estimating guanine is described which depends upon the quantitative conversion of guanine to guanidine by oxidation with permanganate and hydrochloric acid and the colorimetric estimation of the guanidine with Marston's reagent.

The application of the method to the estimation of nucleic acid in animal tissues is also described.

It is proposed to utilize the method for the estimation of nucleo-cytoplasmic ratios, nucleic acid nitrogen being employed as a measure of relative nuclear masses and coagulable protein nitrogen as a measure of relative masses of cytoplasm.

ACKNOWLEDGMENT.

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PRELIMINARY STUDY UPON THE NUCLEO-CYTOPLASMIC RATIO IN PLANT TISSUES

by

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INTRODUCTION.

The nucleo-cytoplasmic ratio has acquired an importance in the fundamental basis of growth, which appears to be controlled by a nuclear autocatalyst. The nucleic acid content of the plant is also of value as representing one of the means of utilization of the phosphoric acid taken up by the plant.

The nucleo-cytoplasmic ratio was measured by the ratio:

$$\frac{\text{Nucleic Acid Nitrogen}}{\text{Total Coagulable Nitrogen} - \text{Nucleic Acid Nitrogen}}$$

The nucleic acid nitrogen was estimated by Robertson's method, which consists in oxidation with $\frac{N}{2}$ $KMnO_4$ of the Cu purine precipitate (obtained by precipitation with $CuSO_4$ in bisulphite solution), and subsequent extraction of the guanidine (derived from the guanine) and determination by Marston's colorimetric method.

After preliminary work, the nucleo-cytoplasmic ratios of a series of wheat plants in different stages of growth were determined by the method following.

NUCLEO-CYTOPLASMIC RATIO IN WHEAT AT DIFFERENT STAGES OF GROWTH.

Method.

Preparation of Dried Sample. A quantity of wheat is cut and converted to chaff. A large enamel basin is filled with the chaff, which is treated with direct steam for 15 minutes to destroy the enzymes, and then dried in a steam-oven to constant weight. The drying usually takes about 2 days. The dried sample is then ground to a fine powder. About 20 g. of the powder are weighed into an extraction thimble, and extracted first with warm ether for 24 hours, and then with hot alcohol for 24 hours.

Nitrogen in Ether Extract. The ether is distilled off on the water-bath, and the residue digested with 15 g. of $KHSO_4$, 18 cc. of concentrated H_2SO_4, and a little $CuSO_4$. The digestion is generally complete in about 3 hours. The digest, after cooling, is made up to 100 cc.; 5 cc. of this solution is taken, Nesslerized, and made up to 100 cc. It is compared with a standard made by taking 10 cc. of a standard $(NH_4)_2SO_4$ solution, containing 0·1 g. of nitrogen per litre, Nesslerizing, and making up to 100 cc.

Nitrogen in Alcoholic Extract. $\frac{1}{50}$ of the extract is evaporated to dryness. A crystalline deposit usually occurs from this extract, and it may be necessary to make an equeous extract of this deposit. The residue is digested with 1 cc. of hydrolyzing-oxidizing mixture. The microkjeldahl is then Nesslerized, made up to 100 cc., and compared with the same standard as used for estimation of nitrogen in ether extract.

Acetic Acid Extract Nitrogen. The residue from the extractions is transferred to a 150 cc. flask, and extracted 3 times with about 400 cc. each of boiling $\frac{N}{50}$ acetic acid, the extract being each time separated by centrifuging. The combined extracts are evaporated and made up to 1000 cc. Forty cc. of this solution were taken, evaporated, and digested with 1 cc. of hydrolyzing-oxidizing mixture. The cooled digest was Nesslerized, made up to 100 cc., and compared with the standard.

Hydrolysis. The residue from the acetic acid extract is treated with 400 cc. of boiling normal HCl, and refluxed for 8 hours in a brine-bath. The hydrolysate is centrifuged, and the residue extracted 3 times with 400 cc. each of hot water, the extracts being separated each time by centrifuging. The combined hydrolysate and washings were evaporated to about 400 cc.

Precipitation of Purines from the Hydrolysate. Ten per cent. NaOH is added to the hydrolysate till it becomes just alkaline. Forty cc. of 30% $NaHSO_3$ is then added, and the liquid centrifuged. The centrifuged solution is heated to boiling, the flame removed, and 40 cc. of 10% $CuSO_4$ solution added little by little to avoid foaming. The solution should then be boiled for at least 2 minutes. The precipitate, after cooling, is centrifuged in a 100 cc. tube, and washed twice with 100 cc. each of distilled water. The tube should be previously marked with a grease pencil at the level corresponding to 10 cc.

Oxidation of the Purine Precipitate. A jet of distilled water is delivered into the centrifuge tube in such a way as to break up the impacted mass of precipitate and suspend it in 10 cc. of water. One cc. of concentrated HCl is added, and the tube heated in boiling brine. When 4 cc. of $\frac{N}{2}$ $KMnO_4$ have been added the precipitate partly dissolves. It is broken up with a glass rod. 0·05 g. of uric acid is added, and the addition of $\frac{N}{2}$ $KMnO_4$ continued with constant heating, till all suspended uric acid is dissolved. A residue still remains.

Estimation of Guanidine Yielded by Oxidation. The fluid is then evaporated to dryness in a porcelain basin on the water-bath. The residue is extracted with sufficient alkaline alcohol to give an alkaline reaction after being ground up and standing for 15 minutes. About 20 cc. are required. The alkaline alcohol is a mixture of 1 vol. of 10% NaOH and 9 vols. of absolute alcohol. The solution is filtered through a glass crucible, and the residue washed four times with 20 cc. of absolute alcohol each time. The filtrate is made distinctly acid with a mixture of 2 vols. of glacial acetic acid and 8 vols. of absolute alcohol (about 2 cc. are required). It is then transferred little by little into a 50 cc. Erlenmeyer flask and evaporated to dryness on the water-bath, finally washing with a little alcohol.

The residue is then taken up in 10 cc. of $\frac{N}{10}$ HCl, solution being aided by gentle warming on the water-bath, after the flame has been turned off. The solution is coloured. One cc. of 10%NaOH is added, and the solution filtered into a 25 cc. volumetric flask, to remove the brown precipitate. The filtrate is more or less yellow.

A standard is made up by introducing 5 cc. of a 0·180% solution of guanidine carbonate in $\frac{N}{10}$ HCl, corresponding to 0·084% of guanidine nitrogen into a 25 cc. volumetric flask and adding 5 cc. of water and 1 cc. of 10% NaOH.

Five cc. of Marston's reagent are then added to the contents of each flask. The mixtures are allowed to stand for 15 minutes, and then made up to volume and compared in a Leitz colorimeter. A solution of 5 cc. of Marston's reagent diluted to 25 cc. is used to correct for the colour of the reagent itself.

Total Nitrogen. One gramme of the dried wheat is weighed into a Kjeldahl flask, and digested with 15 g. of $KHSO_4$, 18 cc. of concentrated H_2SO_4, and a little $CuSO_4$. The digest on cooling is made up to 100 cc.; 2 cc. of this solution is Nesslerized and made up to 100 cc. It is then compared with the standard.

Calculation.

$$\text{Per cent. total nitrogen} = 5 \times \frac{\text{length of standard}}{\text{length of unknown}}$$

$$\text{Per cent. ether extract nitrogen} = \frac{2}{\text{weight of wheat}} \times \frac{\text{length of standard}}{\text{length of unknown}}$$

$$\text{Per cent. alcoholic extract N.} = \frac{5}{\text{weight of wheat}} \times \frac{\text{length of standard}}{\text{length of unknown}}$$

$$\text{Per cent. acid extract N.} = \frac{2{\cdot}5}{\text{weight of wheat}} \times \frac{\text{length of standard}}{\text{length of unknown}}$$

Per cent. coagulable N. = total nitrogen — (ether extract N. + alcoholic extract N. + acetic acid extract N.)

$$\text{Per cent. nucleic acid N.} = 5 \times \text{guanidine N.}$$

$$= \frac{2{\cdot}1}{\text{weight of wheat}} \times \frac{\text{length of standard}}{\text{length of unknown}}$$

$$\text{Nucleo-cytoplasmic ratio} = \frac{\text{Nucleic acid nitrogen}}{\text{Coagulable nitrogen} - \text{Nucleic acid nitrogen}}$$

RESULTS.

Wheat from variety tests at Waite Institute. Sown 18th May, 1928.
Seed, 60 lbs. per acre. Superphosphate, 100 lbs. per acre.

Sample:				
Plot and date	Plot 3, 16/6/28.	Plot 8, 3/7/28.	Plot 13, 14/7/28.	
Age	29 days.	46 days.	57 days.	
Wheat extracted:				
Weight thimble	3.52	3.56	3.55	3.70
Weight thimble + wheat	13.17	27.06	22.51	25.53
Weight wheat	9.65	23.50	18.96	21.83
Total nitrogen:				
Length of std.	20	20	20	20
„ unknown	17.0	16.3	19.5	19.5
% Total N.	5.88	6.13	5.13	5.13
Ether extract N.:				
Length of std.	15	30	20	20
„ unknown	37.0	20.4	7.6	28.0
% Nitrogen	0.084	0.125	0.278	0.065
Alcoholic ext. N.:				
Length of std.	15	20	20 9.8	20 8.8
„ unknown	27.6	31.7	43.9 50	18.8 50
			0.120 0.052	0.244 0.040
% Nitrogen	0.282	0.134	0.172	0.284
Acetic acid ext. N.:			10 cc. ext.	10 cc. ext.
Length of std.	20	20	30	30
„ unknown	24.2	12.2	41.6	32.5
% Nitrogen	0.214	0.174	0.380	0.423
% Coagulable N.	5.3	5.7	4.30	4.36
				Mean 4.33
Nucleic acid N.:			std. half-strength.	
Length of std.	10	10	3.5	3.2
„ unknown	12.4	12.8	10	10
% Nitrogen	0.175	0.0698	0.0194	0.0154
				Mean 0.0174
Nucleo-cytoplasmic ratio	0.0341	0.0124	0.0045	0.0036
				Mean 0.0040
Remarks	Whitish brown residue after oxidation of purine precipitate with $KMnO_4$.		Very dark solution after oxidation with $KMnO_4$. 15 cc. of $KMnO_4$ required.	

The results obtained in this preliminary investigation are of a provisional character, because they were undertaken before Robertson's method had been completely elaborated, and several sources of possible error, in particular the inhibiting effect of partial oxidation products of purines upon colour development with Marston's reagent, to which reference is made in Robertson's paper

(contained in this issue), were not completely eliminated. The absolute values of the figures obtained are therefore subject to revision, but since these sources of error affected all the estimations alike, and the method employed never varied, there can be no doubt that the *relative* magnitudes of the nucleo-cytoplasmic ratios at the ages studied are correctly represented by these figures. We may conclude, therefore, that in the plant, as in animals, the nucleo-cytoplasmic ratio diminishes very rapidly with age at a comparatively early stage of growth.

SUMMARY.

The nucleo-cytoplasmic ratio has been estimated as the ratio of $\frac{\text{Nucleic acid nitrogen}}{\text{Coagulable nitrogen} - \text{Nucleic acid nitrogen}}$ in young wheat plants by the method of Robertson.

It was found that, as in animals, the nucleo-cytoplasmic ratio diminishes very rapidly with age at a comparatively early stage of growth.

SEASONAL VARIATION IN THE IODINE PERCENTAGE AND DRY WEIGHT OF THE THYROID GLANDS OF SHEEP IN [redacted] AUSTRALIA

by

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(Submitted for publication 14th February, 1929.)

As part of a programme of investigation which is being carried out by the Animal Products Research Foundation, in collaboration with the Division of Animal Nutrition of the Commonwealth Council for Scientific and Industrial Research, the iodine contents of the thyroid glands of sheep throughout Australia are being determined. This work falls into two parts.

In the first place it is essential that we should determine approximately the amount of iodine in the thyroid glands of sheep all over the country. To do this only small numbers of glands from any one place are analysed, but they are collected over very wide areas.

The second part of the investigation consists in analysing large numbers of glands from a few selected sheep stations scattered about Australia.

The present paper contains a short summary of the results obtained on analysing 106 thyroid glands, collected, through the courtesy of the owner, Mr. R. G. Beggs, from merino sheep at the sheep station, "Buln Gherin," near Beaufort, in the Western District of Victoria. None of these sheep received any salt lick.

The analysis of one hundred or more glands from one spot makes it possible to determine the degree of variation in iodine percentage and dry weight, and to plot distribution curves.

METHOD.

The technique employed was as follows: The thyroid glands were dissected out of the sheep immediately after slaughtering the animals, and were placed in absolute alcohol in small phials, which were sent to Adelaide to be analysed. Accompanying each phial was a description of the sheep from which the gland was obtained, stating the breed, age and sex of the animal, its physical condition,

and the condition of its wool, and giving information concerning the land on which it was pastured and the water received.

Prior to analysis the glands were freed from any connective tissue and fat which had not been removed in the field, cut into pieces, and, together with the alcohol in which they were preserved, dried to constant weight in a hot-water jacketed oven at a temperature of approximately 92°C.

The method used for the iodine estimation was that of Hunter (1). The 0·005 normal solution of sodium thiosulphate used in the final titration was standardized against a standard solution of 0·005 normal potassium iodate as follows. With each batch of thyroid glands analysed two small quantities of casein were fused and oxidized in exactly the same way as the glands. After dissolving the fused mixtures in water, and transferring the solutions to two flasks, 20 cc. of a standard solution of 0·005 normal potassium iodate were added to each. The solutions were then chlorinated by the addition of sodium hypochlorite solution, acidified, and boiled in exactly the same way as the solutions of the oxidized glands.

Potassium iodide was added to all the solutions in the usual way, and the liberated iodine titrated with an approximately 0·005 normal solution of sodium thiosulphate. In this way the iodate used to standardize the thiosulphate was carried through the same process as the iodine from the glands, so that any error introduced by the process of chlorinating, acidifying, and boiling would have the same effect upon this potassium iodate solution as upon the solution of iodine from the glands.

Blank experiments with casein were carried out from time to time, and the maximum amount of iodine liberated at the end was equivalent to 0·1 cc. of 0·005 normal sodium thiosulphate solution. Usually no iodine was liberated on the addition of the potassium iodide.

Known amounts of potassium iodide added to the mixture of salts, before or after fusion, were recovered quantitatively.

Owing to the small size of the majority of the glands the iodine estimations could not be done in duplicate. However, on two occasions composite samples of dry ground-up thyroid glands were analysed.

In the first case, out of seven analyses, the most extreme divergence from the average was 1·3% of the average, and the average divergence was 0·5%.

In the second case, out of nine analyses, the most extreme divergence from the average was 0·6% of the average, and the average divergence from the average was 0·25%.

RESULTS.

In the following charts and tables the most important results obtained are summarized.

Chart 1 is a distribution curve for the iodine percentages of 106 thyroid glands.

Table 1 gives the percentage of iodine in the individual thyroid glands which go to make up Chart 1.

It will be noticed that Table 1 has been drawn up so that it corresponds in shape with the chart. The percentages of iodine expressed in any column of the table are those of the glands which go to make up the corresponding column in the chart.

In the same way each chart has a table of the same number to correspond with it.

The distribution curve in Chart 1 is bimodal. When, however, the glands are divided into two series, containing approximately equal numbers, according to the time of the year when they were collected, two unimodal curves are obtained (Charts and Tables 2 and 3). The curve for the glands collected between March and the end of August has a mode between 0·538 and 0·609% iodine, while for those collected during September, October, and November the mode is between 0·395 and 0·466% iodine. These correspond with the two modes on Curve 1. The average percentage of iodine for all the glands is 0·5128, for those collected between March and the end of August, 0·5812, and for those collected during September, October, and November 0·4331.

There is thus a fall of 25% in the average iodine percentage of the glands collected during September, October, and November as compared with those obtained during the previous six months.

The number of glands collected during single months was too small to furnish reliable averages for such variable material, but when the average percentage of iodine for the glands collected during each month was considered it was found that the marked fall in iodine percentage occurred about the end of September. For statistical purposes, however, the glands collected during September (eleven in number) have been included with those collected in October and November, rather than in the first series, because in this way the numbers of the glands in the two series are much more nearly equal.

Siedell and Fenger (2) have reported a similar fall in the iodine percentage of sheeps' glands in the corresponding months of March, April, and May in the United States of America.

It is interesting to notice that a similar seasonal variation exists in Australia, and in a locality where the iodine percentage of the glands is much higher than that contained in the glands investigated by Seidell and Fenger. The iodine percentage of the thyroid glands of sheep analysed by them, calculated to a dried basis, varied between 0·042 and 0·335.

In Charts and Tables 5 and 6 the average iodine percentage of the glands of the ewes only is considered. Owing to the smaller number of glands available the total range of iodine percentages is divided into five parts instead of ten. The same shifting of the mode with the season is shown here, the average iodine

percentage falling from 0·5837 to 0·4742 during the spring and early summer months. The average iodine per cent. for all the ewes' glands is 0·5019.

In Chart and Table 4 we have the distribution curve for the dried weights of the same 106 glands.

In this case no simplification is effected by dividing the glands into two series according to the season of the year.

However, in Charts 7 and 8 the distribution curves for the dried weights of the ewes' glands in the periods March to August and September to November are shown. There is a distinct increase in the weight of the glands in the second period, the average rising from 0·5926 grammes to 0·6651 grammes, a rise of 12·2%. This rise in the weight of the glands is not so great as the fall in the iodine percentage, i.e., 18·8%. It follows that the fall in iodine percentage during the spring and early summer months cannot be wholly accounted for by increase in the dry weight of the gland.

Siedell and Fenger (2) report a similar seasonal variation in the weight of thyroid glands.

It should be noticed that while the average iodine percentage of the glands of the ewes is approximately the same as that for all the glands (ewes, wethers, rams, and stag rams), the average dried weight is not. The average dried weight of all the glands is 0·7436 grammes, while that for the ewes is 0·6351 grammes.

The result is that the preponderance of male animals in the period March to August (19 ewes to 38 male sheep), as compared with the period September to November (27 ewes to 22 male sheep) completely masks the rise in the weight of the glands in the latter period. As a matter of fact, when all the glands are considered there is a fall in the average weight of the glands in the second period. In order to show the rise in the weight of the thyroid glands corresponding to the fall in the iodine percentage, with the season of the year, sheep of the same sex should be considered. Ewes were chosen for the purpose, as they were evenly distributed between the two periods.

SUMMARY.

A study of the dry weight and iodine content of 106 thyroid glands collected during nine months from sheep pastured upon one property in the Western District of Victoria, Australia, shows that there is a distinct seasonal variation in the iodine percentage. On plotting the distribution curve of iodine percentage it was found that the curve for all of the glands is bimodal, but on segregating the glands into two groups, the one collected in the Australian spring and early summer months, September, October, and November, and the other in the preceding six months, the two groups yield unimodal curves, the average iodine percentage of the glands collected during September, October, and November

being 25% less than the average for glands collected in the preceding six months. There is a corresponding but less marked rise in the dried weight of the glands.

REFERENCES.

1. Hunter, A.: Journ. Biol. Chem., 7 (1909-10), p. 321.
2. Seidell, A. and Fenger, F.: Journ. Biol. Chem., 13 (1912-13), p. 517.

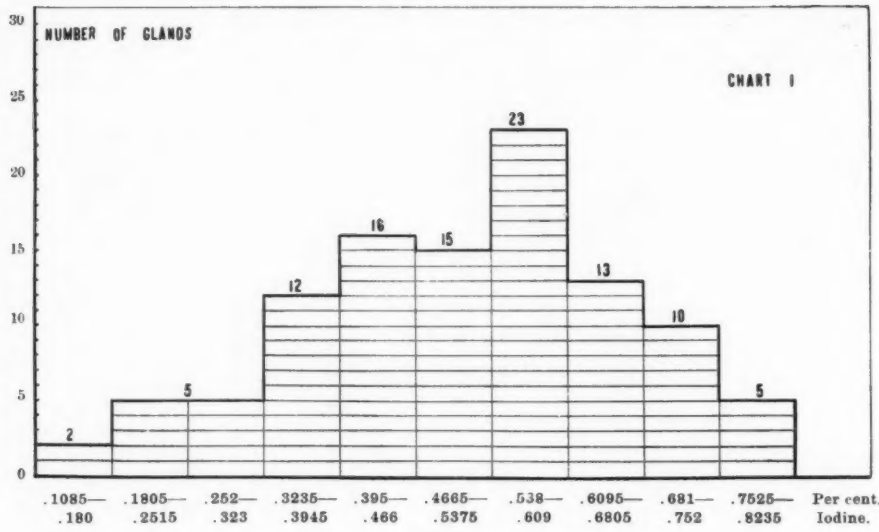

Chart 1.

Distribution curve of iodine percentage in 106 thyroid glands of sheep collected at "Buln Gherin," near Beaufort, in the western district of Victoria, between March and November, 1928.

Average per cent. iodine = .5128.

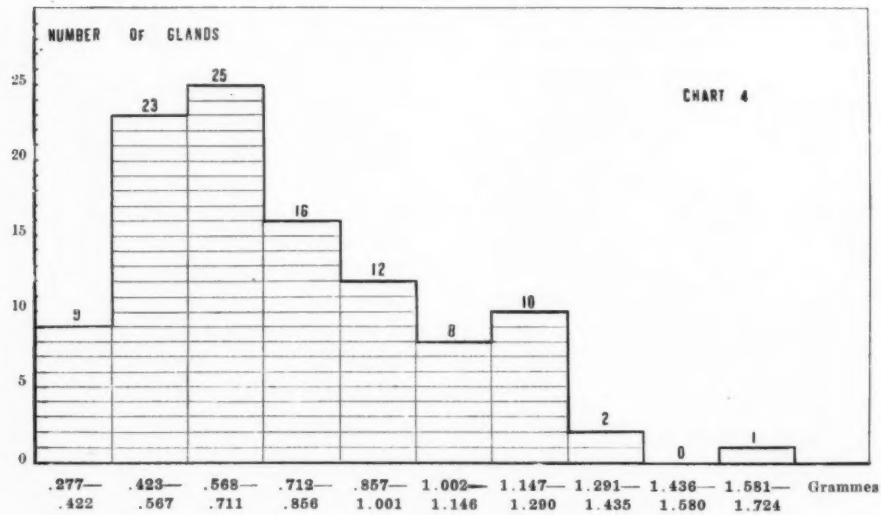

Chart 4.

Distribution curve of dried weights of 106 thyroid glands of sheep collected at "Buln Gherin," near Beaufort, in the western district of Victoria, between March and November, 1928.

Average weight = .7436 grammes.

Average per cent = .5128

						.567				
						.541				
						.609				
						.576				
						.588				
						.568				
						.595				
					.460	.605				
					.404	.483	.597				
					.434	.523	.566				
					.444	.492	.608	.648			
				.326	.424	.497	.591	.677			
				.373	.457	.485	.565	.669			
				.364	.438	.511	.595	.644	.740		
				.334	.413	.526	.567	.642	.695		
				.340	.447	.5065	.550	.629	.694		
				.382	.439	.494	.569	.637	.732		
				.344	.424	.489	.586	.676	.686		
	.247	.252	.347	.437	.479	.586	.678	.7345	.817	
	.228	.2995	.366	.415	.475	.539	.619	.722	.800	
	.245	.281	.353	.453	.477	.549	.643	.709	.754	
.147	.212	.287	.360	.402	.528	.601	.619	.723	.803	
.1085	.244	.277	.326	.449	.530	.585	.618	.740	.8235	
.1085—	.1805—	.252—	.3235—	.395—	.4665—	.538—	.6095—	.681—	.7525—	Per cent.
.180	.2515	.323	.3945	.466	.5375	.609	.6805	.752	.8235	iodine.

TABLE 1.

Distribution of Iodine Percentage in 106 Thyroid Glands of Sheep collected at "Buln Gherin," near Beaufort, in the Western District of Victoria, between March and November, 1928.

		.662								
		.687								
	.544	.688								
	.521	.634								
	.562	.609								
	.520	.571								
	.546	.569								
	.512	.710								
	.468	.655				Average weight = .7436 grammes				
	.477	.576	.816							
	.525	.600	.843							
	.501	.618	.815							
	.443	.684	.778							
	.535	.682	.713	.918						
	.516	.701	.719	.985						
	.511	.623	.772	.946		1.251				
.353	.535	.581	.718	.881		1.166				
.307	.519	.616	.824	.896	1.022	1.244				
.410	.515	.589	.770	.924	1.055	1.204				
.325	.428	.653	.777	.890	1.016	1.231				
.277	.480	.595	.741	.875	1.010	1.149				
.327	.531	.600	.765	.930	1.021	1.207				
.393	.554	.611	.765	.935	1.018	1.201				
.422	.520	.630	.733	.894	1.038	1.252	1.341			
.411	.536	.615	.766	.925	1.046	1.175	1.350		1.724	
.277—	.423—	.568—	.712—	.857—	1.002—	1.147—	1.291—	1.436—	1.581—	Grammes
.422	.567	.711	.856	1.001	1.146	1.290	1.435	1.580	1.724	

TABLE 4.

Distribution of Dried Weights of 106 Thyroid Glands of Sheep collected at "Buln Gherin," near Beaufort, in the Western District of Victoria, between March and November, 1928.

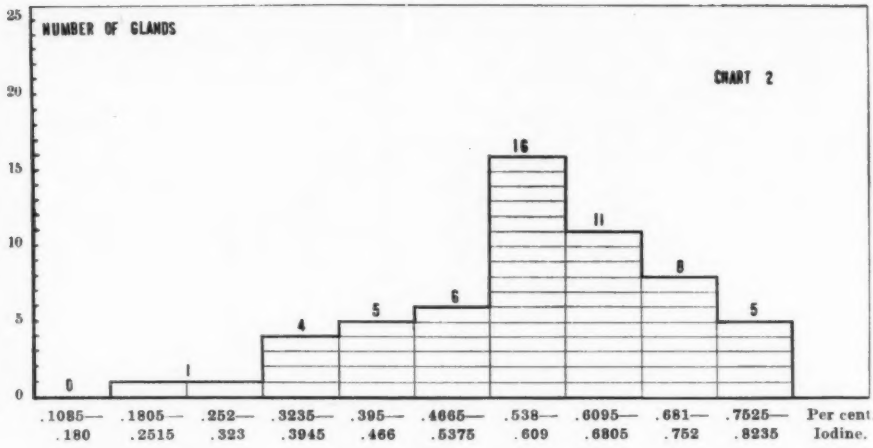

Chart 2.

Distribution curve of iodine percentage in 57 thyroid glands of sheep collected at "Buln Gherin," near Beaufort, in the western district of Victoria, between March and August, 1928.

Average per cent. iodine = .5812.

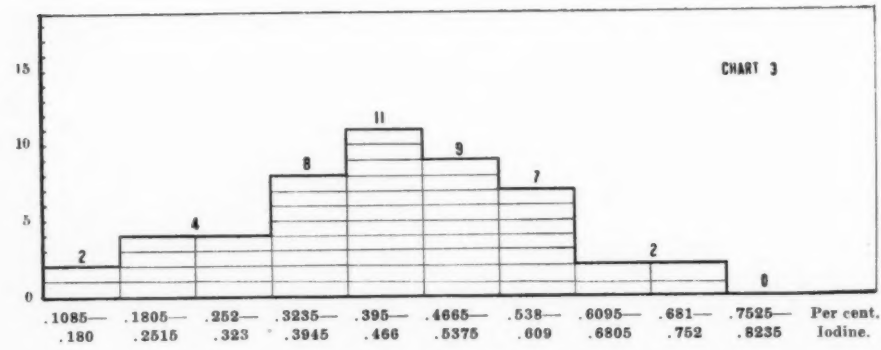

Chart 3.

Distribution curve of iodine percentage in 49 thyroid glands of sheep collected at "Buln Gherin," near Beaufort, in the western district of Victoria, between September and November, 1928.

Average per cent. iodine = .4331.

						.605				
						.597				
Average per cent. = .5812						.566				
						.608				
						.591				
						.565	.669			
						.595	.644			
						.567	.642			
						.550	.629	.694		
						.569	.637	.686		
					.489	.586	.676	.7345		
				.437	.479	.586	.678	.732	.817	
			.366	.415	.475	.539	.619	.722	.800	
			.353	.453	.477	.549	.643	.709	.754	
			.360	.402	.528	.601	.619	.723	.803	
	.244	.277	.326	.449	.530	.585	.618	.740	.8235	
.1085—	.1805—	.252—	.3235—	.395—	.4665—	.538—	.6095—	.681—	.7525—	Per cent.
.180	.2515	.323	.3945	.466	.5375	.609	.6805	.752	.8235	iodine.

TABLE 2.

Distribution of Iodine Percentage in 57 Thyroid Glands of Sheep collected at "Buln Gherin," near Beaufort, in the Western District of Victoria, between March and August, 1928.

				.460		Average per cent. = .4331				
				.404						
				.434	.483					
			.326	.444	.523					
			.373	.424	.492	.567				
			.364	.457	.497	.541				
			.334	.438	.485	.609				
	.247	.252	.340	.413	.511	.576				
	.228	.2995	.382	.447	.526	.588				
.147	.245	.281	.344	.439	.5065	.568	.648	.740		
.1085	.212	.287	.347	.424	.494	.595	.677	.695		
.1085—	.1805—	.252—	.3235—	.395—	.4665—	.538—	.6095—	.681—	.7525—	Per cent.
.180	.2515	.323	.3945	.466	.5375	.609	.6805	.752	.8235	iodine.

TABLE 3.

Distribution of Iodine Percentage in 49 Thyroid Glands of Sheep collected at "Buln Gherin," near Beaufort, in the Western District of Victoria, between September and November, 1928.

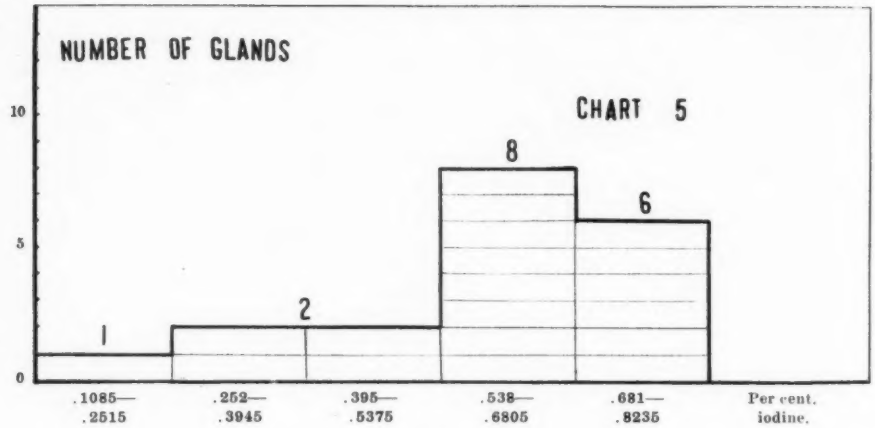

Chart 5.

Distribution curve of iodine percentage in thyroid glands of 19 ewes collected at "Buln Gherin," near Beaufort, in the western district of Victoria, between March and August, 1928.

Average per cent. iodine = .5837.

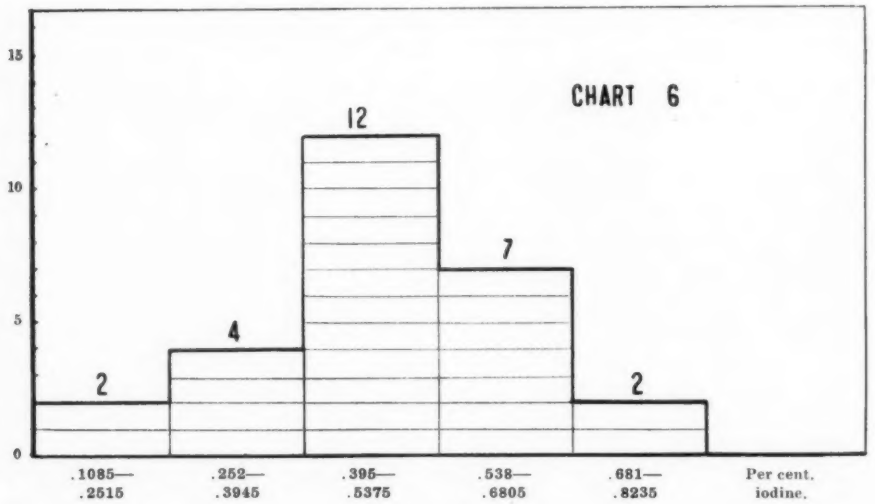

Chart 6.

Distribution curve of iodine percentage in thyroid glands of 27 ewes collected at "Buln Gherin," near Beaufort, in the western district of Victoria, between September and November, 1928.

Average per cent. iodine = .4742.

			.669		
			.629		
			.605	.817	
Average per cent. = .5837			.597	.694	
			.566	.686	
			.608	.734	
	.353	.489	.591	.732	
.244	.277	.528	.549	.722	
.1085—	.252—	.395—	.538—	.681—	Per cent.
.2515	.3945	.5375	.6805	.8235	iodine.

TABLE 5.

Distribution of Iodine Percentage in Thyroid Glands of 19 Ewes collected at "Buln Gherin," near Beaufort, in the Western District of Victoria, between March and August, 1928.

		.483			
		.497			
		.526	Average per cent. = .4742		
		.506			
		.494			
		.460	.677		
		.444	.567		
		.424	.609		
	.334	.438	.576		
	.347	.447	.588		
.245	.252	.424	.568	.740	
.147	.281	.439	.595	.695	
.1085—	.252—	.395—	.538—	.681—	Per cent.
.2515	.3945	.5375	.6805	.8235	iodine.

TABLE 6.

Distribution of Iodine Percentage in Thyroid Glands of 27 Ewes collected at "Buln Gherin," near Beaufort, in the Western District of Victoria, between September and November, 1928.

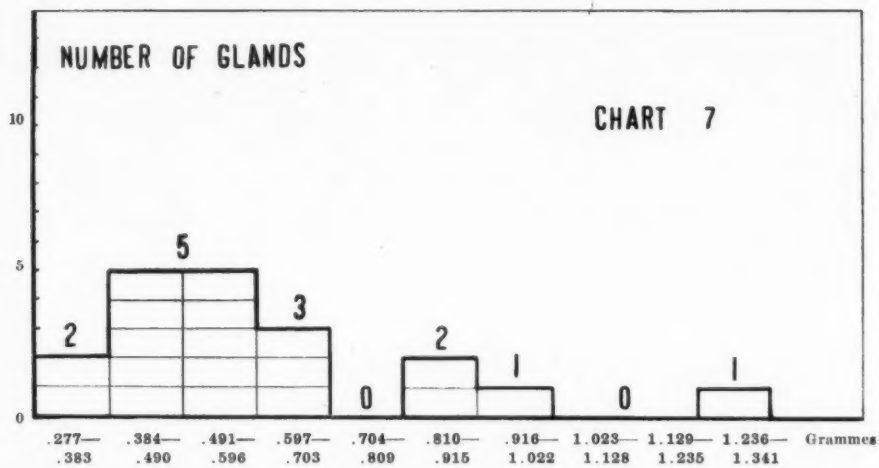

Chart 7.

Distribution curve of dried weights of thyroid glands of 19 ewes collected at "Buln Gherin," near Beaufort, in the western district of Victoria, between March and August, 1928.

Average weight = .5926 grammes.

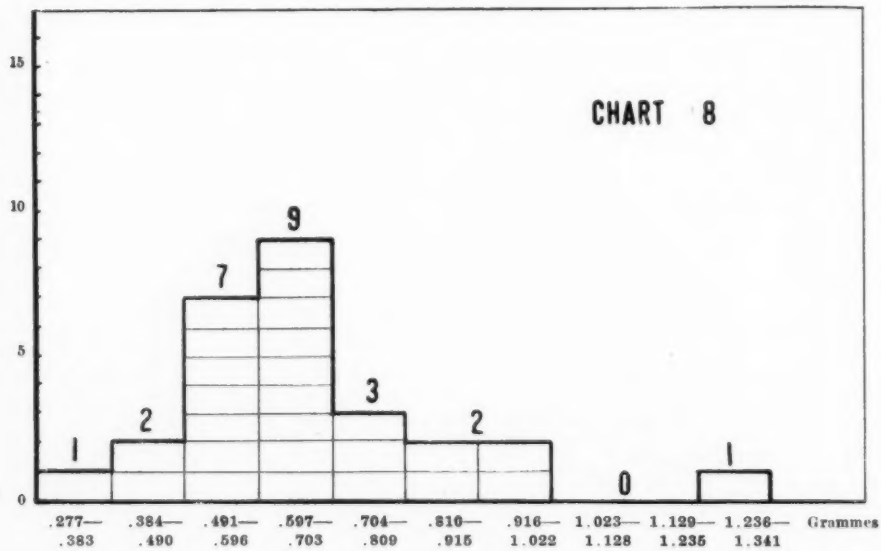

Chart 8.

Distribution curve of dried weights of thyroid glands of 27 ewes collected at "Buln Gherin," near Beaufort, in the western district of Victoria, between September and November, 1928.

Average weight = .6651 grammes.

	.428	.581								
	.410	.511				Average weight = .5926 grammes.				
	.393	.535	.623							
.325	.422	.519	.616		.881					
.277	.411	.536	.630		.896	.924			1.341	
.277—	.384—	.491—	.597—	.704—	.810—	.916—	1.023—	1.129—	1.236—	Grammes
.383	.490	.596	.703	.809	.915	1.022	1.128	1.235	1.341	

TABLE 7.

Distribution of Dried Weights of Thyroid Glands of 19 Ewes collected at "Buln Gherin," near Beaufort, in the Western District of Victoria, between March and August, 1928.

			.687							
			.688							
		.576	.609							
		.544	.655			Average weight = .6651 grammes.				
		.562	.600							
		.520	.618							
		.501	.684	.713						
	.477	.535	.682	.777	.843	.918				
.353	.443	.516	.701	.741	.824	.946			1.244	
.277—	.384—	.491—	.597—	.704—	.810—	.916—	1.023—	1.129—	1.236—	Grammes
.383	.490	.596	.703	.809	.915	1.022	1.128	1.235	1.341	

TABLE 8.

Distribution of Dried Weights of Thyroid Glands of 27 Ewes collected at "Buln Gherin," near Beaufort, in the Western District of Victoria, between September and November, 1928.